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URINARY INDOLEACETIC ACID

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "URINARY INDOLEACETIC ACID", submitted by Stephen Harold in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Utilizing a reaction that converts indole-3-acetic acid (IAA) into a highly fluorescent indolopyrone derivative, a new specifc and sensitive method for the estimation of IAA in urine has been developed.

A study of the fluorescence and absorption characteristics of the reagents and reaction products was undertaken. Included was a study of the effect of light, solvent media, concentration of reagents, pH, temperature, time and order of addition of reagents.

The application of the method to urine samples involved extraction efficiency studies and the effect of acid and alkaline hydrolysis on the liberation of IAA from its conjugated forms.

Extracts of urine were found to contain substances which caused quenching of the fluorescence reaction. Attempts to remove the interfering substances with charcoal, ion-exchange resins, Florisil, alumina, etc. were unsuccessful. A method using an internal standard was finally adopted to overcome the interference.

A normal IAA urinary excretion of 1.5 to 18.4 mg. per 24 hours or 2.9 to 12.0 mg. per g. creatinine was obtained using the new method. Preliminary studies of the IAA excretion in several disease states showed that there was

no consistent abnormal excretion in patients with malabsorption or muscular dystrophy. Two patients with phenylketonuria had elevated IAA excretion.

The colorimetric method of Weissbach, et al. (1) was run in parallel with the new method on all estimations performed on urine. It yielded significantly higher free and significantly lower total IAA values than the fluorimetric method. An investigation showed that the high free IAA values were probably due to the non-specificity of the colorimetric technique. The low total IAA values obtained with the colorimetric technique may be attributed to the partial destruction of IAA in the conjugated form by acid hydrolysis. The colorimetric method of Weissbach, et al. (1) was found to be less precise than the fluorimetric method with a standard deviation for the difference between duplicates of 0.76 (for the former) compared with 0.26 (for the latter).

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I. INTRODUCTION

Indole-3-acetic acid (IAA) is a metabolite of tryptophan. It may be formed either by transamination of tryptophan to indole-3-pyruvic acid and subsequent decarboxylation or by decarboxylation to tryptamine followed by oxidative deamination of tryptamine by monoamine oxidase (see Figure 1). Weissbach, King, Sjoerdsma, and Udenfriend (1) found that many tissues are capable of forming IAA from tryptophan but Tezuka (2) did not observe formation in liver and kidney homogenates of mice and rats. Intestinal bacteria also can form IAA from tryptophan (2) but only via the indole-3-pyruvic acid pathway since they are unable to metabolize tryptamine (1).

IAA is excreted daily in small amounts in the urine. Increased urinary excretion of IAA in malabsorption has been reported by Weissbach, et al. (1) and by Haverback, Dyce, and Thomas (3). The latter workers attributed the increased IAA excretion to a change in the intestinal flora since sterilization of the intestinal tract, as carried out by Weissbach, et al. (1), substantially reduced the urinary IAA but did not lower the fecal fat. Contrary results were reported by Marko and Gerrard (4) who, using the method of Weissbach, et al. (1), found no increase in 26 cases of malabsorption in adults and children. Increased urinary excretion of IAA has been found also in mental and other diseases (5,6,7,8,9,10). Most investigators used the method of Weissbach, et al. (1) for the estimation of IAA (3,4,11,12,13,14). The occurrence of contradictory reports suggested the possibility that the methods for

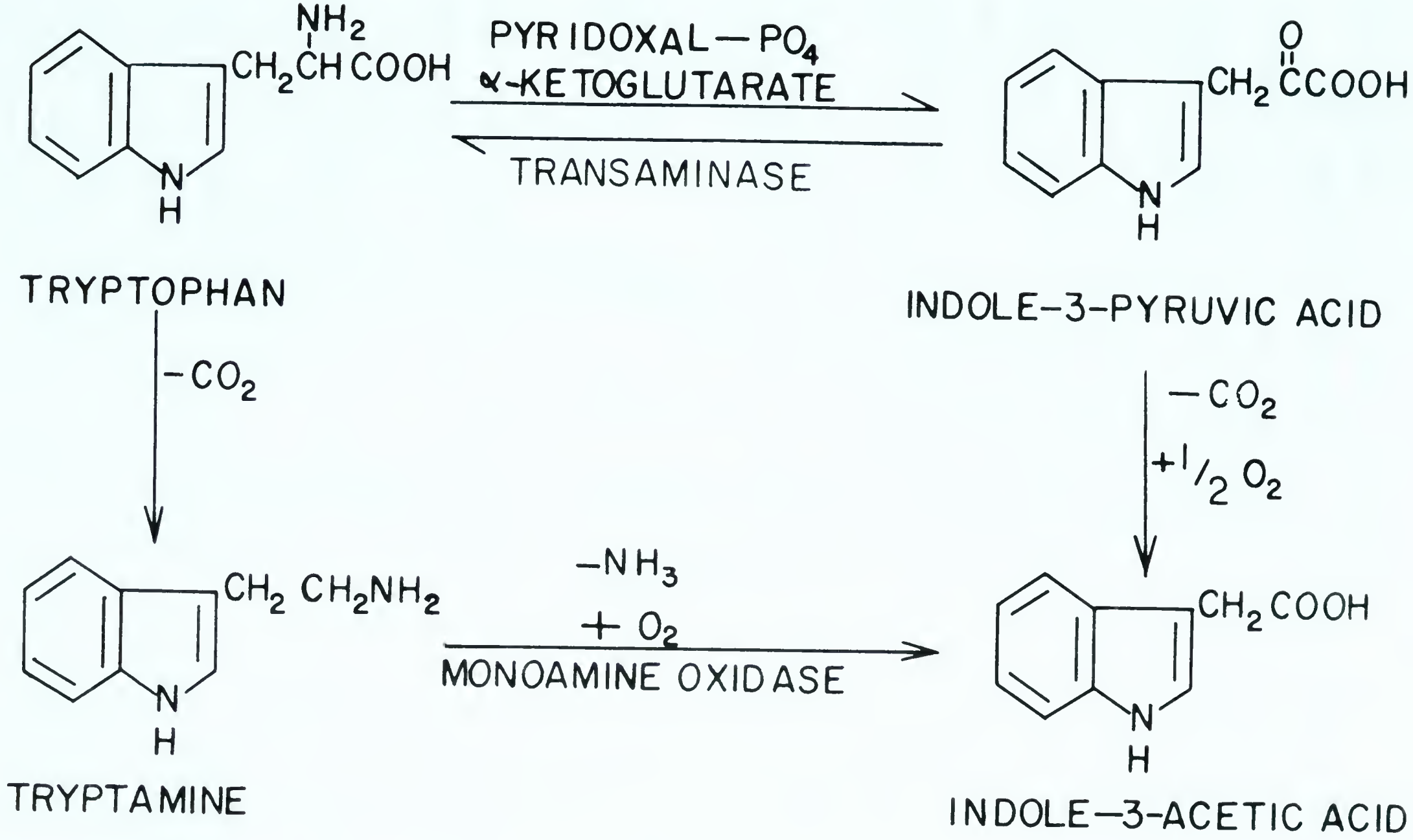


Fig. 1. Indole-3-acetic acid formation from tryptophan in mammalian tissue and fecal bacteria (1).

the estimation of IAA were inadequate.

The estimation of IAA by the method of Weissbach, et al. (1) consists of solvent fractionation using chloroform and phosphate buffer at pH 7.0, followed by colorimetric estimation of the product of the reaction between IAA and xanthydrol and bisulfite. This method appears to lack specificity since any chloroform soluble indole could interfere. Other colorimetric procedures (15,16,17,18) lack sensitivity and employ lengthy chromatographic separations to provide specificity. Measurement of the native fluorescence of IAA in chloroform extracts of tissues has been used for IAA estimations but this procedure is not applicable to urine because of the presence of interfering substances. There appeared to be a need for a sensitive and more specific method for the estimation of IAA in urine. The development of such a method forms the basis of this thesis.

Initial consideration of the problem suggested that fluorimetry would have certain advantages. It is more (about 1000 times) sensitive and more specific than absorptimetry. Improvement in specificity results from the fact that fluorescence measurements are based on two (exciting and emitting) wavelengths compared with the single wavelength which characterizes a substance in absorption methods. A successful method, based on the formation of fluorescent indolopyrone derivative by a reaction first described by Plieninger and Müller (19), was finally attained. This new method was adapted to measuring the urinary excretion of IAA by normal subjects and subjects with malabsorption, muscular dystrophy and phenylketonuria.

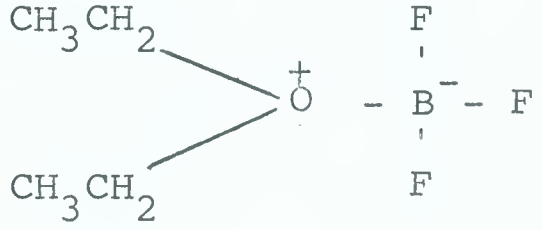
II. METHODOLOGY

1. THE FLUORESCENCE REACTION

a. Chemical equations for the Plieninger-Müller reaction

Plieninger and Müller, in a preliminary report published in 1960 (19), described the formation of a fluorescing derivative of IAA when IAA is treated with boron trifluoride ethyl ether ($BF_3.Et_2O$) in acetic anhydride-ethyl ether solution at room temperature. The reactions and the proposed resonant structures are shown in Figure 2 (19).

$BF_3.Et_2O$ is a complex between a Lewis Acid (BF_3) and base (ethyl ether), the structure of which is shown below:



IAA is acetylated in the presence of Ac_2O and the ring closure is catalyzed by $BF_3.Et_2O$, as shown in Figure 2.

The resulting yellow compound gave an intense green fluorescence in water and a deep red solution in dilute alcoholic sodium hydroxide. The yellow compound was given the name "indolo-(2',3';4,5)-6-methylpyrone-2". This compound will subsequently be referred to as indolopyrone or the indolopyrone derivative (of IAA).

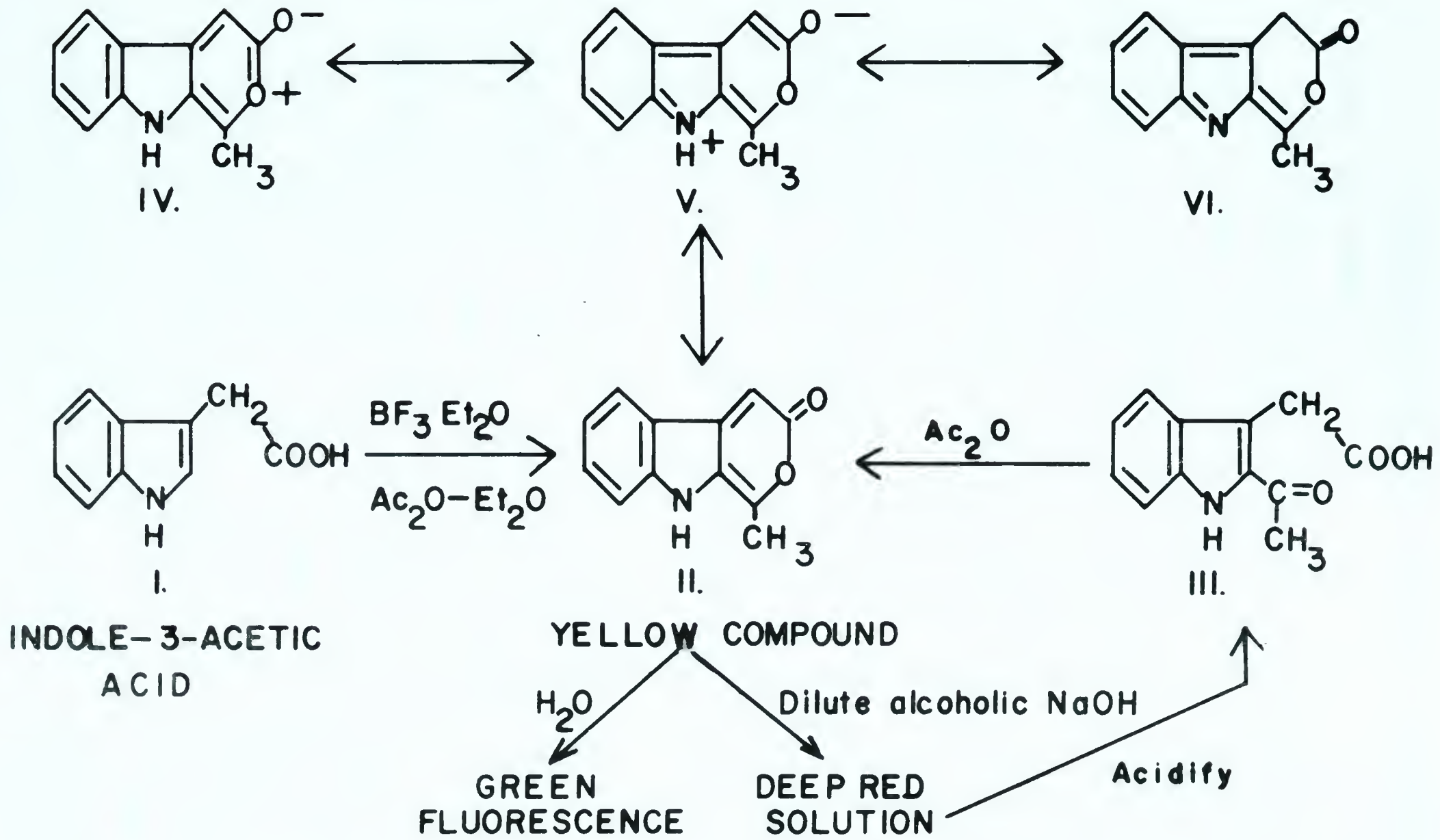


Fig. 2. Chemical reaction of indole-3-acetic acid with $BF_3 \cdot Et_2O$ and acetic anhydride in ethyl ether and resultant resonant structures (~~25~~ 19).

b. Activation and fluorescence spectra of the reaction mixture and reagents

The above reaction appeared promising as an analytical method for IAA and initial experiments were carried out as follows. In the presence of $BF_3.Et_2O$ and using ethyl ether as reaction solvent, 1 mg. of IAA and 1 ml. of acetic anhydride reacted to form a yellow compound. The yellow compound exhibited a brilliant yellow-green fluorescence under short wave ultraviolet light. Evaporation of the ether and addition of water produced a green fluorescence. A preliminary study showed that this reaction had a high degree of specificity for IAA in that other urinary indoles, with the exception of 5-hydroxyindoleacetic acid (5-HIAA), did not yield a fluorescent product.

The Aminco-Bowman Spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Maryland) and the Beckman DK-2 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, California), were used to study the reaction.

The activation and fluorescence spectra were first determined. Activation is the process by which a molecule absorbs light energy and is raised to a higher electronic energy level. An activation spectrum is obtained when the incident light from the primary monochromator is varied through the wavelength range of interest while the secondary monochromator is fixed at the peak fluorescence wavelength. Fluorescence is the light energy emitted when the excited molecule returns to its original energy level in 10^{-8} seconds or less following activation. The fluorescence spectrum is obtained in a similar manner except

that the secondary monochromator is varied through the wavelength range while the primary monochromator is fixed at the wavelength of maximum activation.

Early in this work it was found that the $BF_3.Et_2O$ reagent also fluoresces and could possibly cause interference. However, a study of the activation spectrum of this reagent in ether showed that its fluorescence was minimal at an activation of 310 mμ which was the point of maximum activation of the indolopyrone derivative. The reaction mixture consisted of IAA, 0.1 ml. Ac_2O and 0.5 ml. $BF_3.Et_2O$ made up to a volume of 10 ml. with ethyl ether. The reagent blank was the same except that IAA was not present.

The activation and fluorescence spectra of the reaction mixture, as well as the fluorescence spectrum of the reagent blank are shown in Figure 3. In anhydrous ethyl ether there were three maxima of activation, namely at 255, 310 and 450 mμ. Activation at 255 mμ gave a low fluorescence response while activation at 450 mμ was too close to the fluorescence maximum of 475 mμ to be useful. The maximum at 310 mμ was chosen for the activation of the indolopyrone reaction product. It can be seen from this diagram that the background fluorescence due to reagents was low. The concentration of IAA in the reaction mixture was 0.5 micrograms per ml. and the settings on the spectrophotofluorometer were as follows:

Slit arrangement #3 (according to the instruction manual accompanying the instrument).

Photomultiplier microphotometer meter multiplier set at 0.3 and sensitivity set at zero.

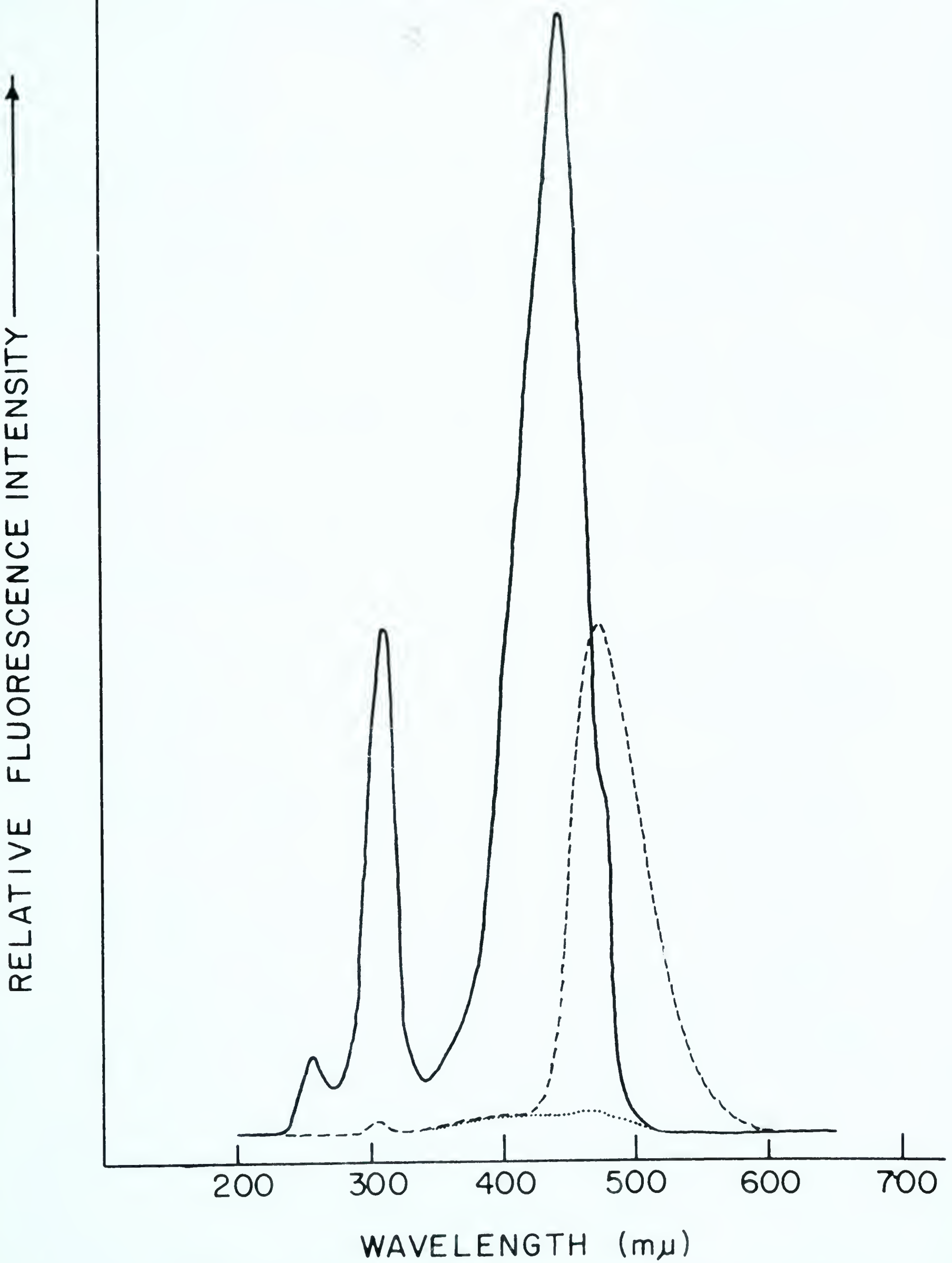


Fig. 3. Activation spectrum of reaction mixture (indolopyrone) in ethyl ether at λ_F =475 mu ——— . Fluorescence spectrum of reaction mixture at λ_A = 310 mu ----- . Fluorescence spectrum of reagent blank at λ_A =310 mu .....

RCA IP 21 (type 931-A) phototube was used throughout this work.

Fluorescence and activation recordings were made on a Moseley Autograph Model 1 X-Y Recorder, F.L. Moseley Co., Pasadena, California.

The fluorescence produced at 475 mμ in the above reaction was approximately 40 arbitrary units (a.u.) on a meter graduated from 0 to 100.

c. Relative intensity of fluorescent product in anhydrous ethyl ether and water.

Since Plieninger and Müller (19) described only the intense green fluorescence of the reaction product (indolopyrone) in water and preliminary work showed that the indolopyrone in the original ether reaction mixture also fluoresced intensely, it was decided to compare the relative fluorescence of the indolopyrone in both media. This was accomplished by first carrying out the reaction in ether. The reaction mixture consisted of 1.0 ml. IAA standard (10 μg./ml. ethyl ether), 0.1 ml. Ac_2O, 0.5 ml. $BF_3.Et_2O$ and 8.4 ml. ethyl ether. After a specified reaction time, part of the solution was transferred to an equal volume of water and the ether evaporated by a stream of air. This brought about the solution of the reaction product. A determination of the activation spectrum on this aqueous solution revealed a shift of the maximum at 310 mμ to 305 mμ, and the fluorescence at the latter activation wavelength was found to have an intensity approximately 1/8th that of the corresponding ether solution. The fluorescence maximum also shifted

from 475 mμ to 470 mμ. In the light of the reduced fluorescence it was decided that an aqueous medium for fluorescence measurement would not be considered further.

d. A study of the reaction in ethyl ether.

Having decided to carry out the reaction and fluorescence measurement in a non-aqueous medium with activation and fluorescence settings of 310 mμ and 475 mμ respectively, the next step was to determine the concentration of reagents that produced maximum fluorescence. It was found that a suitable reaction mixture consisted of IAA in ether, 0.5 ml. $BF_3.Et_2O$ and 0.1 ml. Ac_2O made up to a 10-ml. volume with ethyl ether. A study of the variation of fluorescence intensity with reaction time is shown in Figure 4. At reaction temperature of 20°C the fluorescence value changed with time. However, as this curve is reproducible, it was concluded that this reaction could be used for analysis of a pure solution of IAA by reading at a constant reaction time. This procedure would yield valid analytical results provided that unreacted IAA does not contribute to the fluorescence measurement. As the fluorescence wavelength of IAA in ether is at 335 mμ, well removed from the 475 mμ peak of the indolopyrone derivative (see Figure 5), IAA will not affect the analytical measurement.

e. A study of the reaction in chloroform.

Although ethyl ether was used for the analysis of pure solutions of IAA, it was not suitable for extracting IAA from urine because 5-hydroxyindoleacetic acid (5-HIAA), an inter-

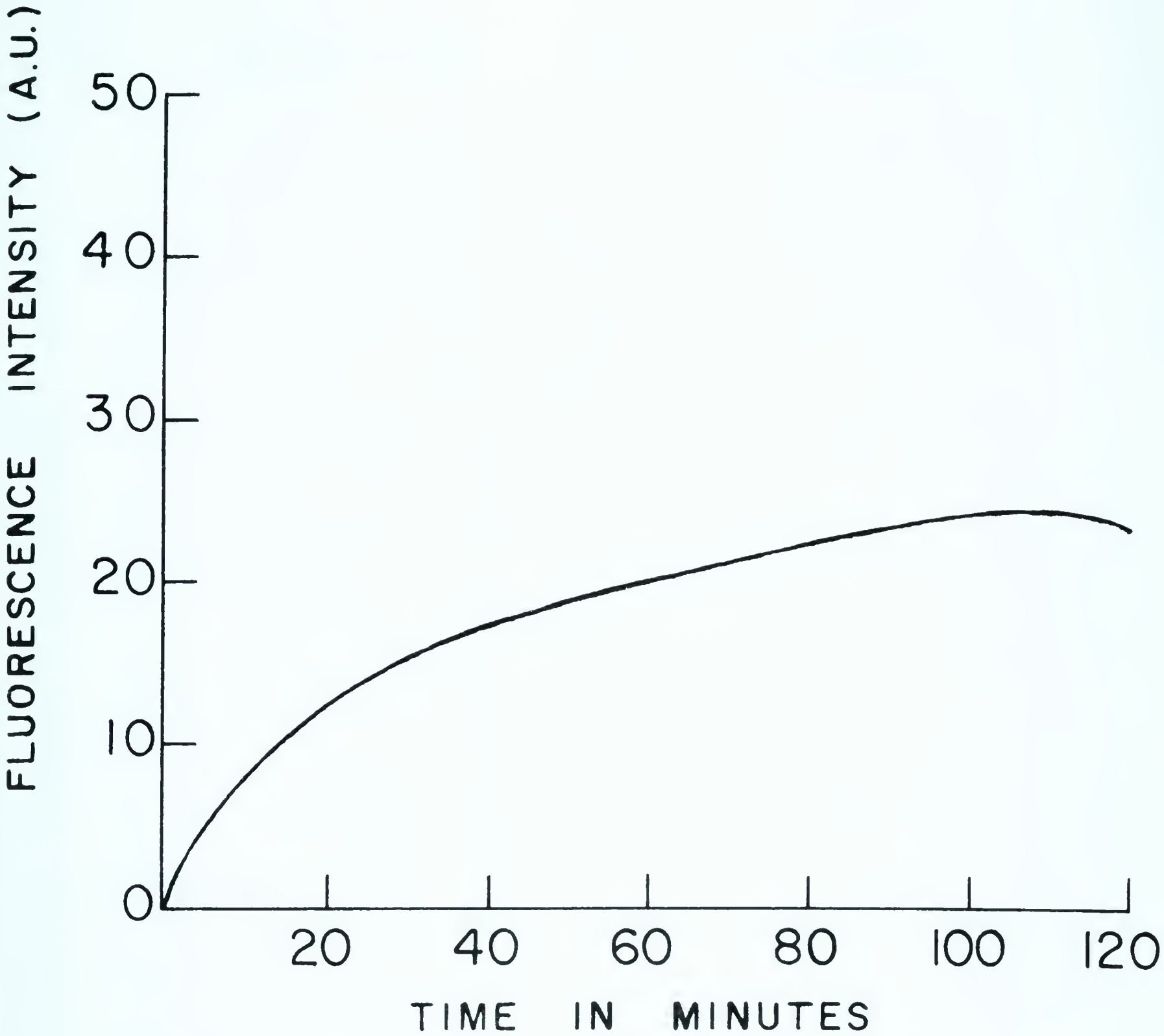


Fig. 4. Variation of fluorescence intensity with reaction time. The reaction mixture consisted of 0.5 ml. IAA standard (10 ug./ml. in ethyl ether), 0.5 ml. $BF_3.Et_2O$, 0.1 ml. Ac_2O, and 8.9 ml. ethyl ether. Activation and fluorescence settings are 310 and 475 mu, respectively.

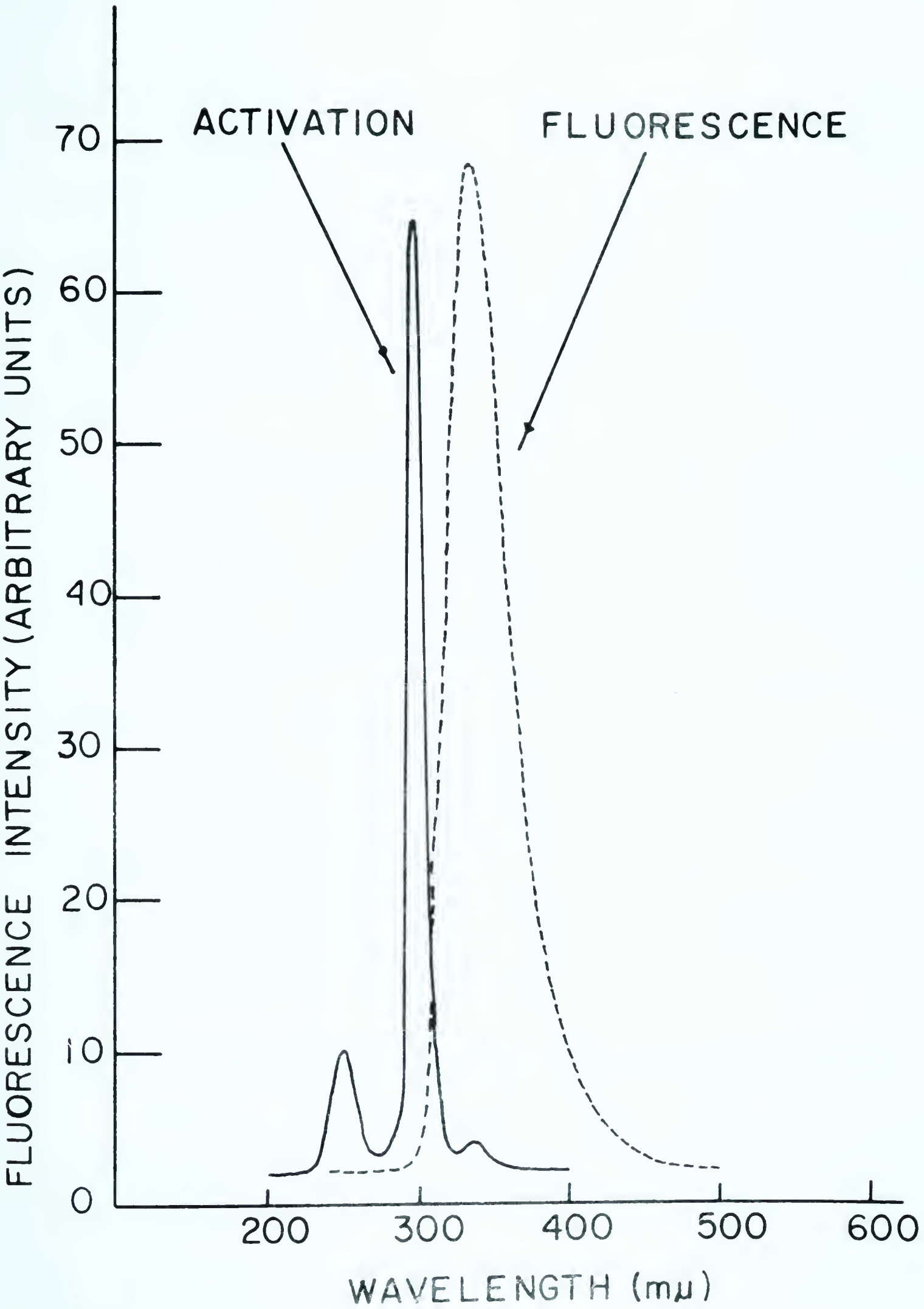


Fig. 5. Activation and fluorescence spectra of indole-3-acetic acid in ethyl ether.

fering indole, was also extractable with ether. In referring to the procedure of Weissbach and associates (1), it was noted that at one step in the procedure, the IAA was extracted into chloroform. This step separated IAA from 5-HIAA in an efficient manner. Thus it was of interest to know whether the IAA fluorescence reaction could be carried out in chloroform solution.

A parallel run was carried out in duplicate standard solutions with 10 ml. of anhydrous ethyl ether as the solvent for one and 10 ml. of reagent grade chloroform as the solvent for the other. After allowing 30 minutes for the reaction, the the chloroform solution gave a significantly greater fluorescence than the ether solution.

Activation and fluorescence studies of the chloroform reaction mixture showed spectra that were very similar to that in ether solution (see Figure 3). The activation maximum was 320 mμ and the maximum for fluorescence was 485 mμ compared to 310 mμ and 475 mμ in ethyl ether.

f. Reaction in chloroform containing ethyl ether.

During the work up to this point, IAA had been made up in anhydrous ether. Standards now were made up in $CHCl_3$ to match the reaction solvent. However, at one point, when working with chloroform as the reaction solvent, a working standard of IAA in ether was used. Significantly higher fluorescence readings were observed. It was thought at first that IAA standards might be unstable in $CHCl_3$. However, stability studies showed that IAA in chloroform was stable for 2 months when kept in the refrigerator. The enhanced fluorescence in

ether-$CHCl_3$ mixtures was studied by reacting 0.5 ml. of IAA standard in $CHCl_3$ (10 μg./ml.) at room temperature as follows:

1/	2/	3/
0.5 ml. standard	0.5 ml. standard	0.5 ml. standard
8.0 ml. $CHCl_3$	8.0 ml. $CHCl_3$	7.0 ml. $CHCl_3$
0.1 ml. Ac_2O	0.1 ml. Ac_2O	0.1 ml. Ac_2O
0.4 ml. $BF_3.Et_2O$	0.4 ml. $BF_3.Et_2O$	1.0 ml. Et_2O
		0.4 ml. $BF_3.Et_2O$

At 30 minutes 1.0 ml. of $CHCl_3$ was added to each of the reaction mixtures 1 and 3, 1 ml. of ethyl ether was added to reaction mixture 2, and the fluorescence intensity of each was measured. The results are shown in Table I.

TABLE I

The effect on the fluorescence intensity of 10% ethyl ether in the chloroform medium

Reaction	Fluorescence intensity (a.u.) (corrected for blank reading)
1	28.3 (no ether present)
2	47.5 (ether added after reaction completed)
3	52.0 (ether in reaction mixture)

It can be seen from these results that the presence of ether during the reaction or added after its completion significantly increased the intensity of the fluorescence.

2. A STUDY OF OTHER FACTORS INFLUENCING THE FLUORESCENCE INTENSITY

a. Fluorescence intensity as a function of water content.

Subsequent studies, in which ether was incorporated into the reaction mixture, revealed that at times variable intensities of fluorescence were obtained with fixed amounts of IAA. It was thought that this might be due to varying amounts of peroxides and/or water in the ethyl ether. To study these effects, reactions in peroxide-free ether (20) containing amounts of water varying from 0.01 to 0.6% were carried out. (During this study it was found that the addition of 2 ml. of peroxide-free ethyl ether to the reaction medium gave maximum fluorescence with good reproducibility.) Ether solutions containing 0.01, 0.1, 0.15, 0.2, 0.4 and 0.6% water were prepared and when 2-ml. portions were used in separate reaction mixtures, fluorescence readings of 55.7, 56.7, 58.0, 56.4, 55.3 and 45.9 arbitrary units (a.u.) respectively were obtained. Maximum reproducible fluorescence was obtained by adding 2 ml. peroxide-free ether containing 0.15% water into 8 ml. of reaction mixture.

b. Absorption spectrum of the reagents in different solvent media.

The absorption spectrum of the reagents in different solvent media was also investigated with and without the presence of ethyl ether in an attempt to explain the effect of ether on fluorescence intensity. Figure 6 shows that ethyl ether affects the absorption characteristics throughout most of the spectrum from 250 to 550 mμ. In both the 320 and 475 mμ region there was

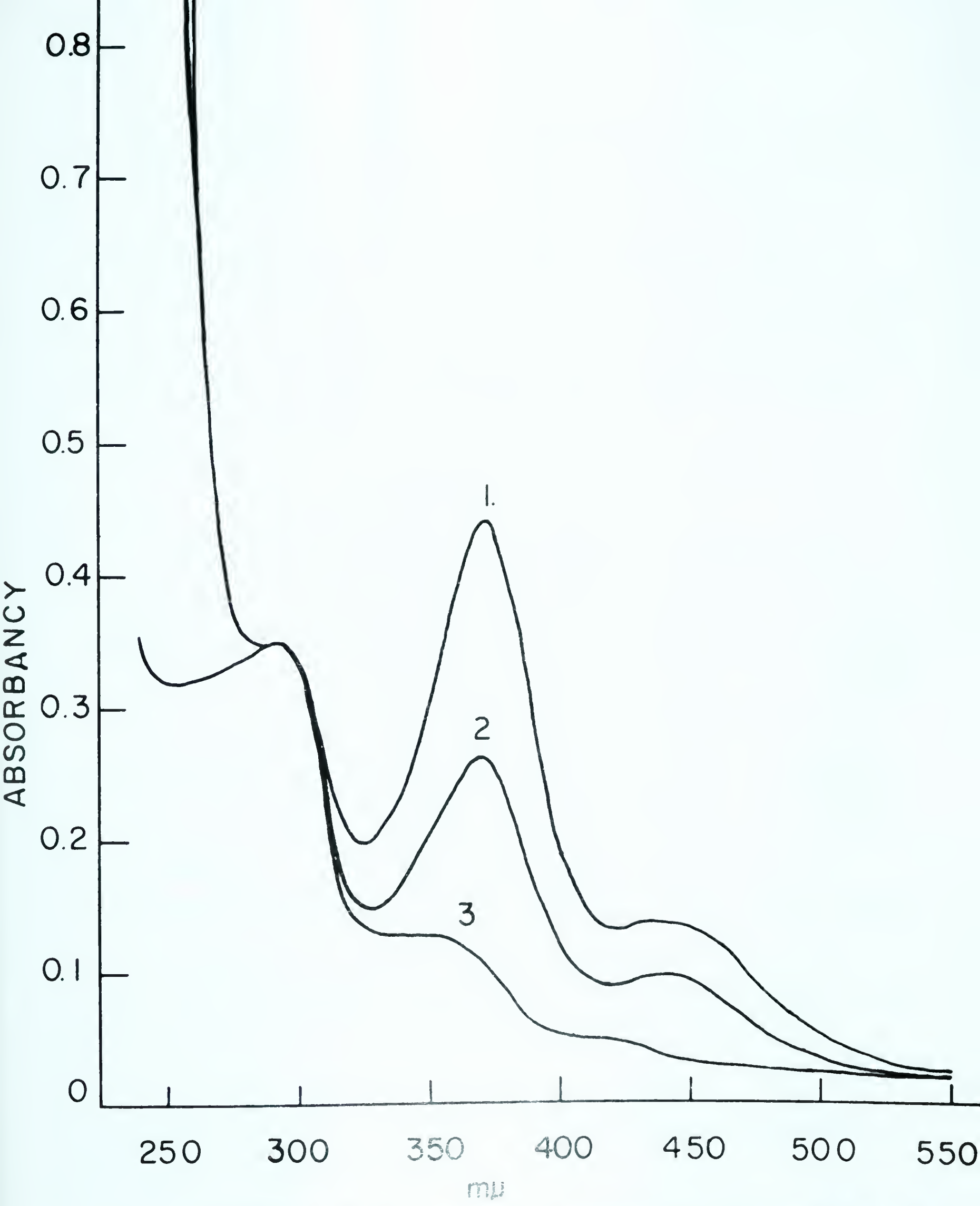


Fig. 6. Effect of ethyl ether on the absorption spectrum of the reagent-solvent mixture. Ether was added to a reagent-solvent mixture consisting of 0.1 ml. propionic anhydride and 0.3 ml. $BF_3.Et_2O$ in $CHCl_3$ in the following amounts. (1) none; (2) 2.0 ml.; (3) 9.6 ml. A total volume of 10.0 ml. was maintained by adjusting the volume with $CHCl_3$.

a decrease of absorbance. These decreases in optical density are small and may only partially explain the marked increase in fluorescence intensity in the mixed solvent. It should be noted that the activation peak is positioned in a "well" of the reagent absorption spectrum resulting in minimal background absorbance of activation energy. Propionic anhydride was used here in place of acetic anhydride. This change in procedure will be discussed next.

c. Effect of anhydride.

The optimal concentrations of acetic anhydride and $BF_3.Et_2O$ were determined under conditions involving the inclusion of 2 ml. ethyl ether in a 10-ml. reaction volume with $CHCl_3$ as solvent. It was found that a volume of 0.3 ml. $BF_3.Et_2O$ and 0.1 ml. of acetic anhydride would yield maximal fluorescence. The activation and fluorescence spectra were again determined for this new solvent mixture and slight changes in the maxima were observed. The maxima activation peak was 316 mμ and maximum fluorescence at 480 mμ. The shape of the curves otherwise are similar to those obtained in Figure 3 where ethyl ether was the solvent. A further increase of fluorescence was obtained with the higher homologues of acetic anhydride, i.e., propionic and butyric anhydride. See Figure 7. Propionic anhydride was chosen for the reaction as butyric anhydride had a very unpleasant odor.

The reaction of IAA with propionic anhydride in the presence of $BF_3.Et_2O$ produces a derivative similar to compound II in Figure 2 except that an ethyl group replaces the methyl

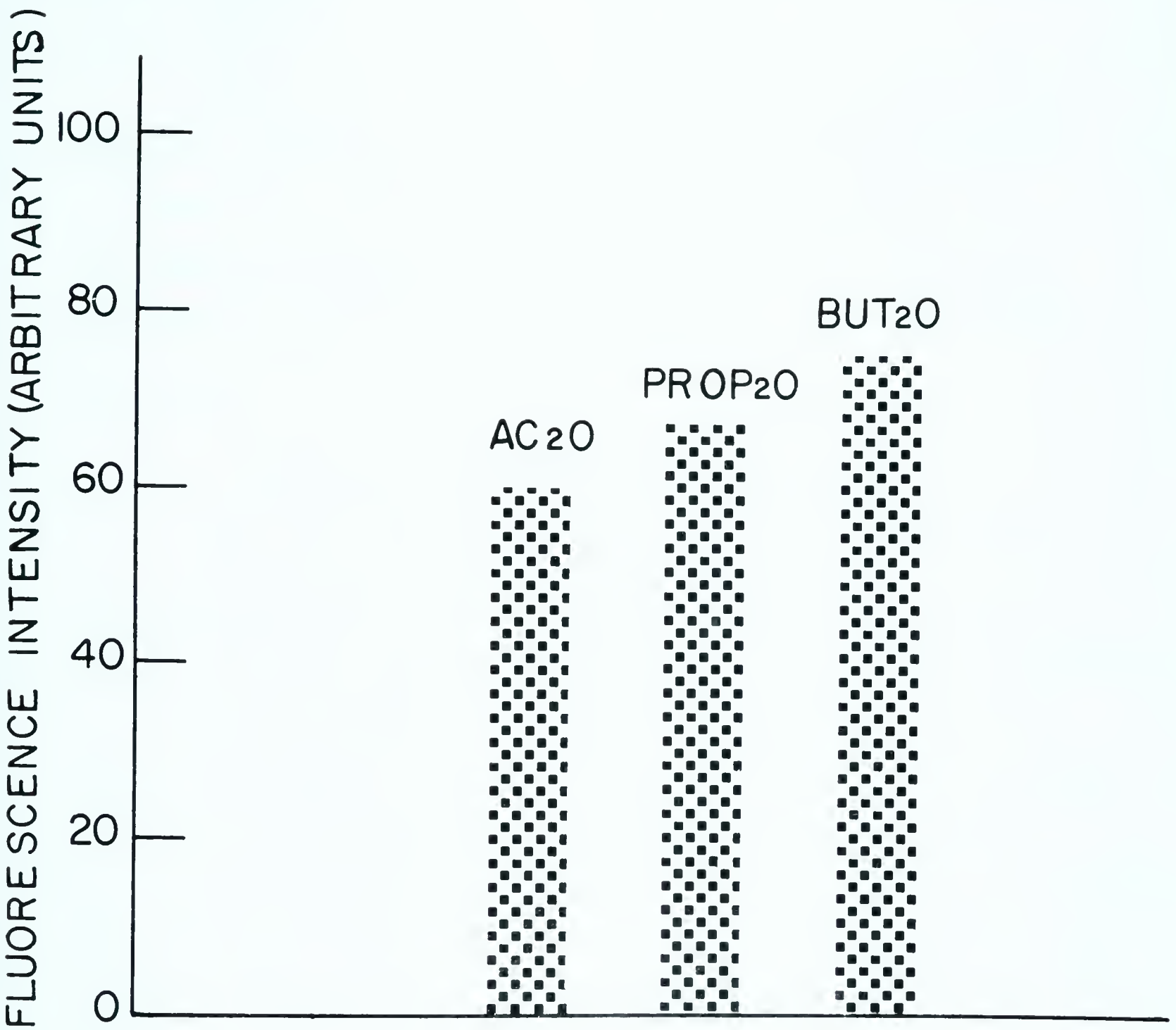


Fig. 7. Effect of anhydrides on fluorescence intensity.

group in position 6. The compound would then be given the name "indolo-(2',3';4,5)-6-ethylpyrone-2".

Studies were also made of any spectral shifts when propionic anhydride is used in place of acetic anhydride. No significant shifts were detected, so no changes in instrument settings were necessary. The only measurable difference appeared to be an increase in the fluorescence intensity.

d. Effect of concentration of $BF_3.Et_2O$.

The following figures show the results that led to the aforementioned optimum reaction conditions. Figure 8 shows the effect of the concentration of $BF_3.Et_2O$ on fluorescence intensity with two different concentrations of IAA, namely 0.2 and 1.0 micrograms per ml. The relative heights of these curves are unimportant since they were read on different sensitivity settings in order to bring them within range of each other. There was no significant shift of the peak with different concentrations of IAA.

e. Effect of concentration of propionic anhydride.

Figure 9 shows the effect of the concentration of propionic anhydride when all other variables were kept constant. Included here are curves illustrating the data obtained when the reaction was allowed to proceed for times of 10, 20, 30 and 40 minutes. At a volume of 0.1 ml. propionic anhydride it can be seen that the reaction reached completion in 30 minutes. This reaction medium consisted of 0.5 μg. IAA, 2.0 ml. ethyl ether, 0.1 ml. $Prop_2O$, 0.3 ml.

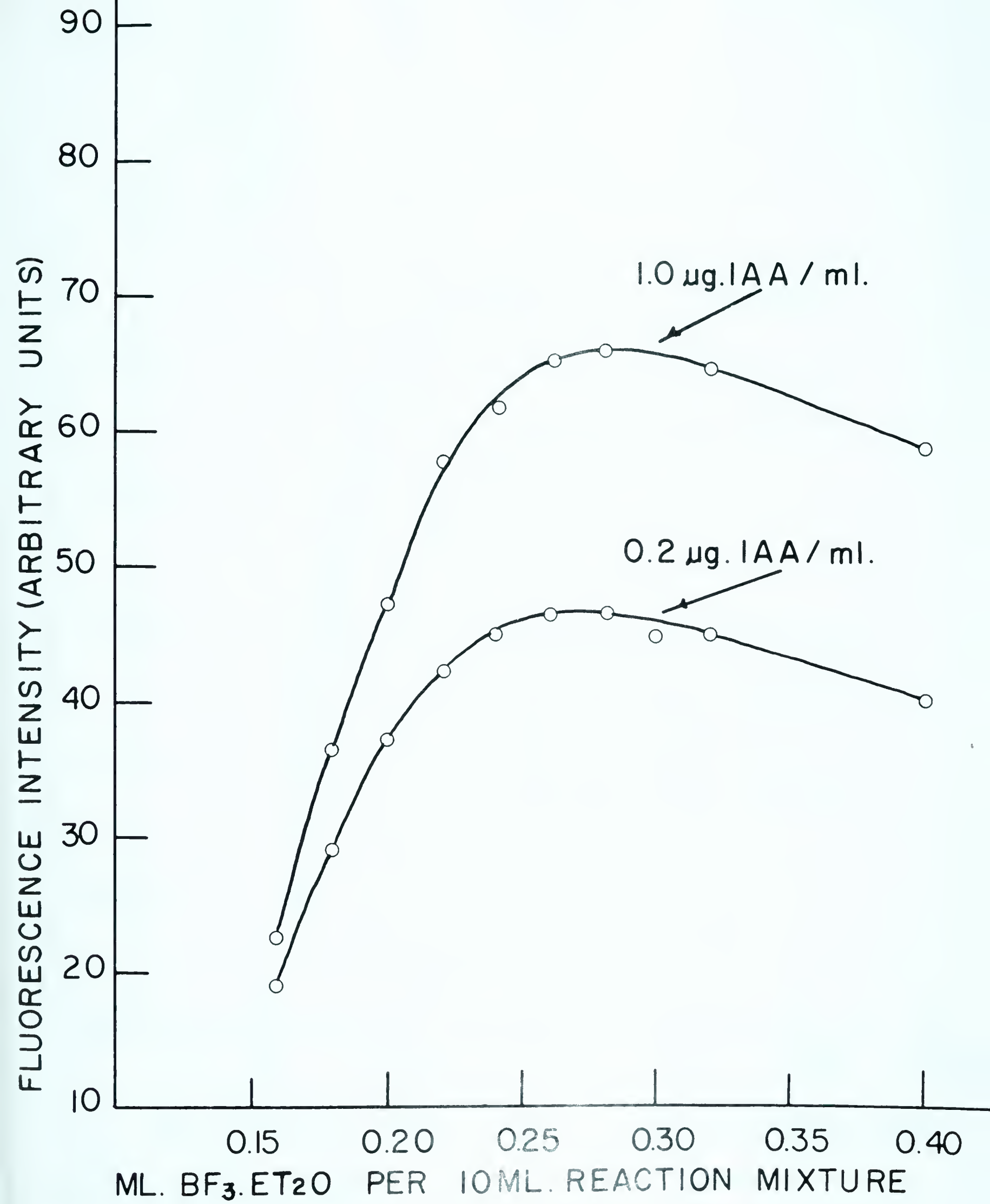


Fig. 8. Effect of concentration of $BF_3 \cdot Et_2O$ on fluorescence intensity at two concentrations of IAA.

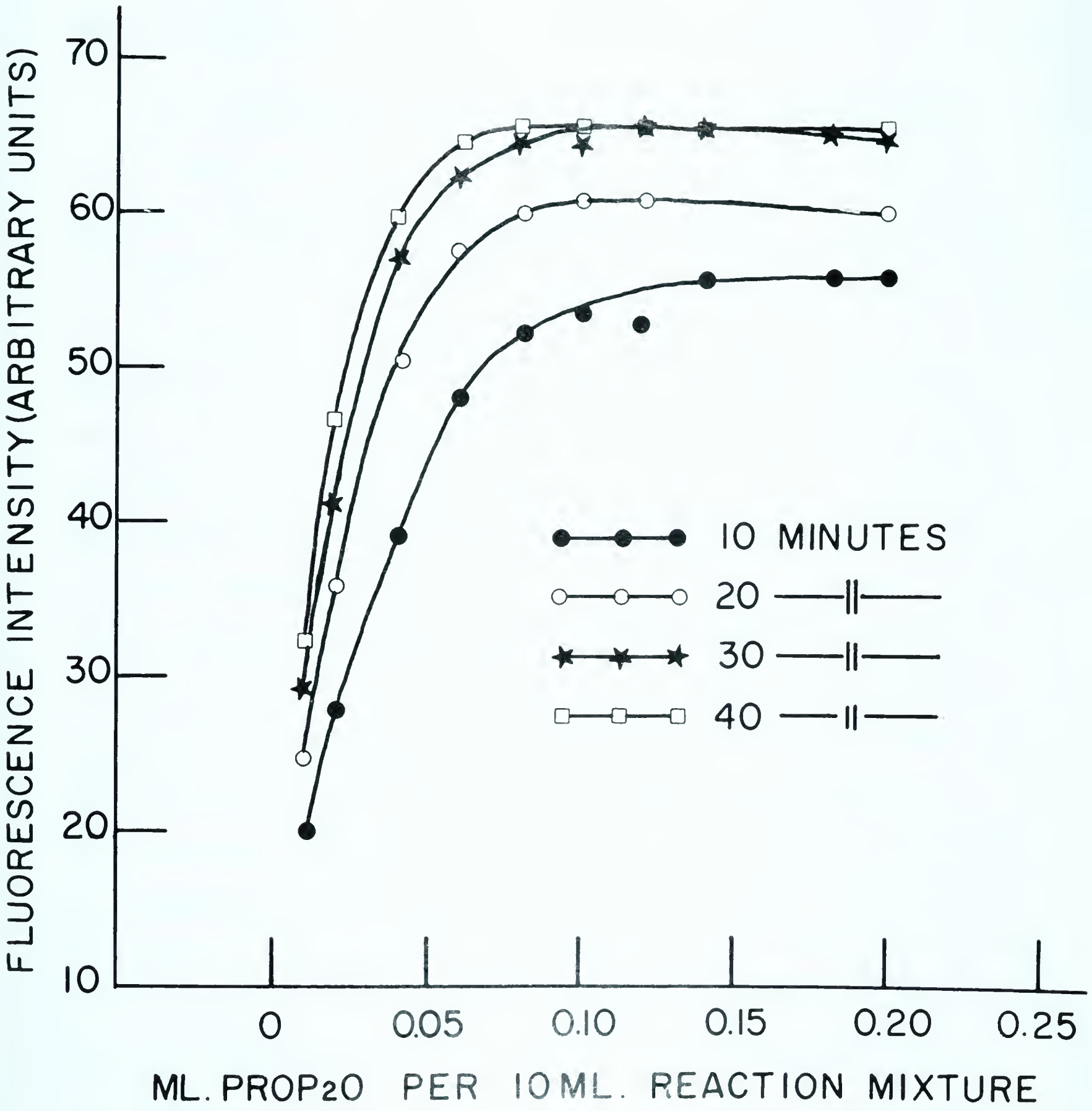


Fig. 9. Effect of concentration of propionic anhydride and the effect of reaction time on fluorescence intensity.

$BF_3.Et_2O$ and $CHCl_3$ to a volume of 10.0 ml. The use of 20% ether in the reaction medium is discussed below.

f. Effect of reaction time and solvent composition.

Figure 10 illustrates the effect of reaction time and solvent composition on fluorescence intensity. Here various percentages of ethyl ether were present in the reaction mixture which included 0.1 ml. Ac_2O, 0.3 ml. $BF_3.Et_2O$, 0 to 5 ml. ether and $CHCl_3$ to a volume of 10 ml. As can be seen, increasing ether concentration delays the time required for the reaction to reach its fluorescence maximum. However, at 30 minutes, a 20% concentration gave the highest fluorescence yield. The optimum concentration of ether is illustrated by Figure 11, in which the fluorescence intensities of a number of reaction mixtures, after a reaction time of 30 minutes, are plotted against the concentrations of ethyl ether in the mixtures. It can readily be seen that a solvent composition containing approximately 20% ether gave maximum value. Acetic anhydride was employed in the experiments from which data illustrated in Figures 10 and 11 were obtained. Essentially identical results with respect to optimal time and ethyl ether concentration were obtained when propionic anhydride was substituted for acetic anhydride.

g. Effect of light.

The effect of light on the intensity of the fluorescence reaction was also studied. This was determined by running duplicate reactions in the light and in the dark. The fluorescence intensity developed was identical.

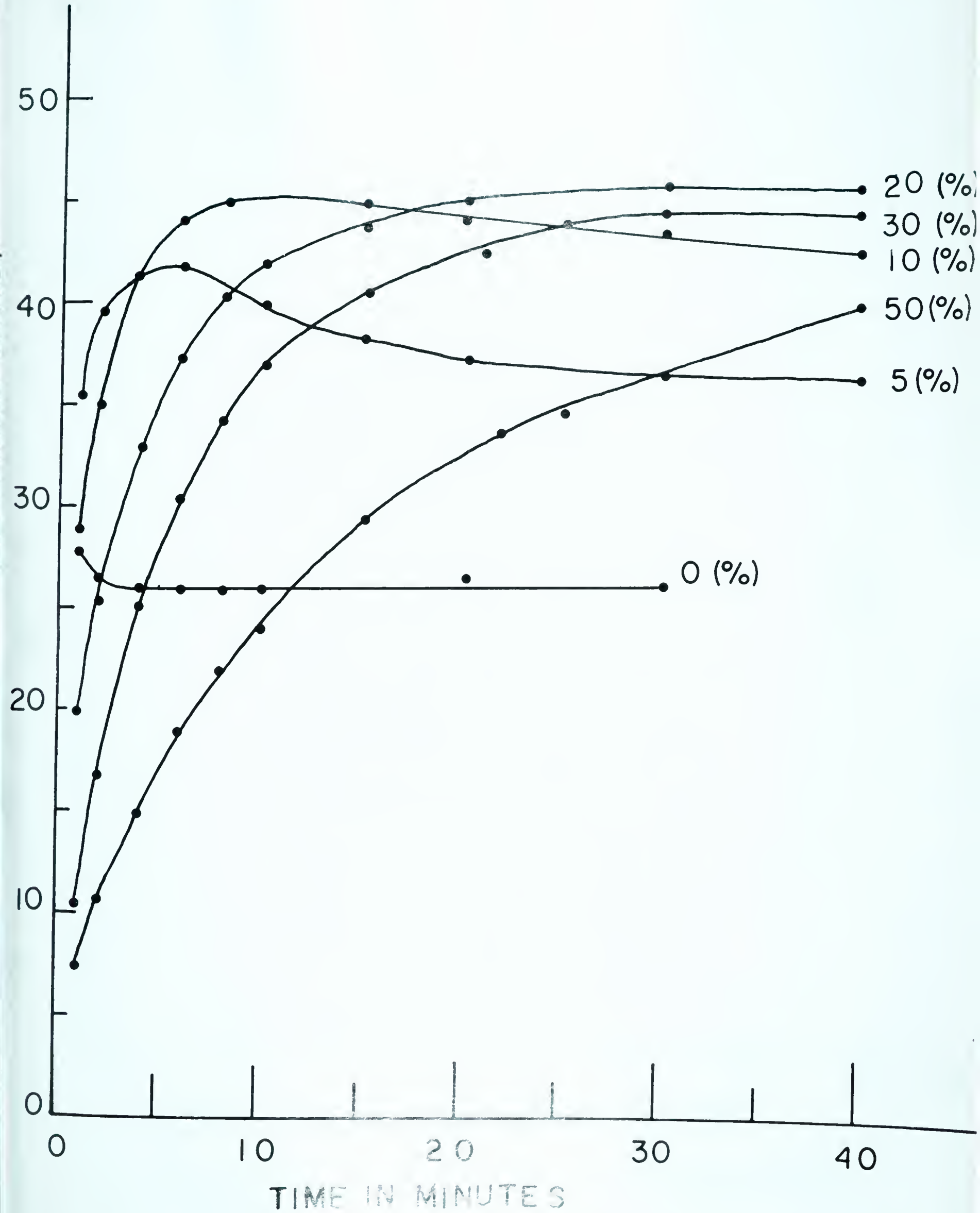


Fig.10. Effect of time and solvent composition on fluorescence intensity. Percentage values represent amount of ethyl ether present in the reaction mixture.

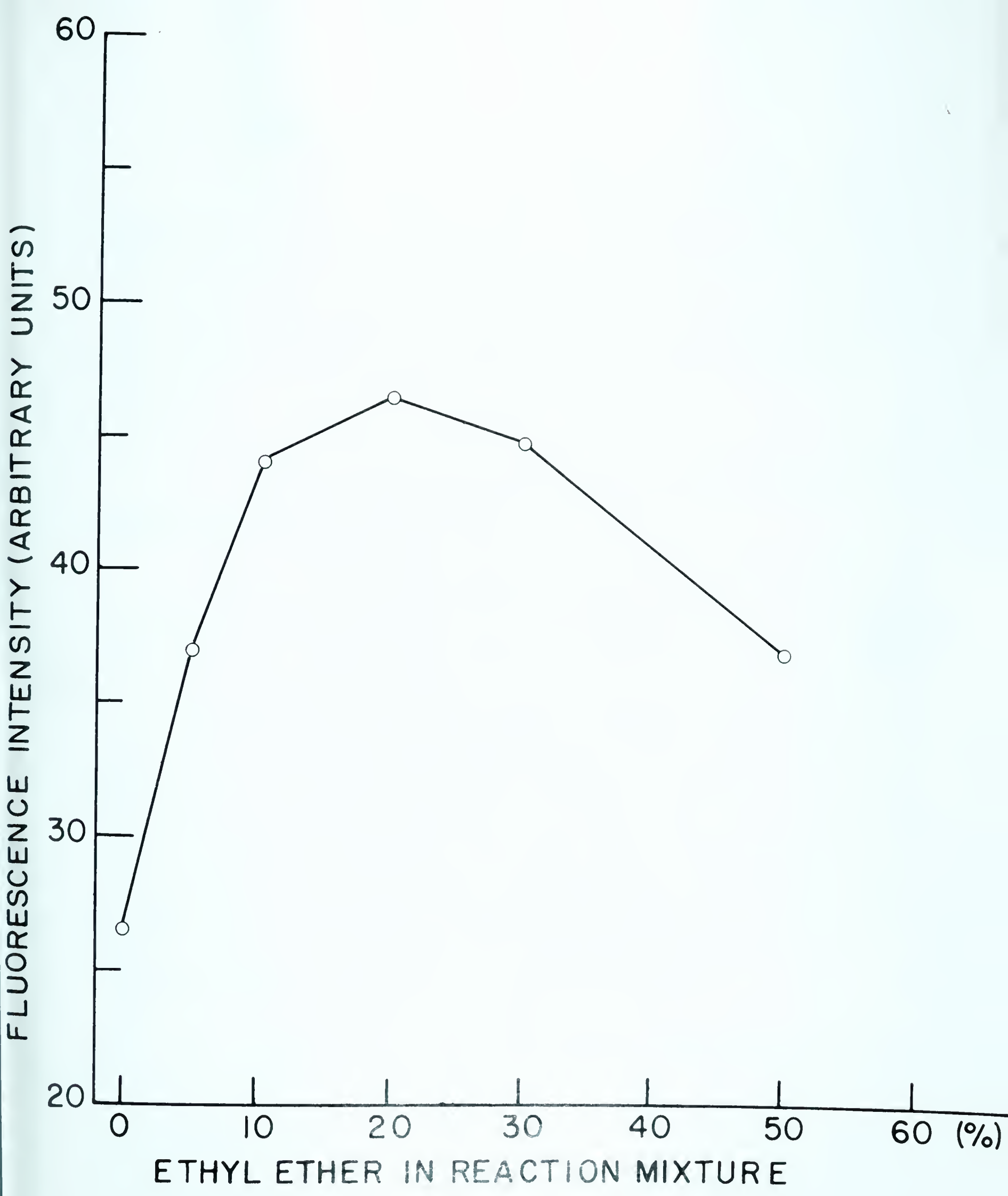


Fig. 11. Effect of solvent composition on fluorescence intensity. points at 30 minutes reaction time from Fig. 9 are re-plotted against percent ethyl ether in the reaction mixture.

h. Effect of temperature.

In order to determine the effect of changes in room temperature on the final fluorescence intensity, the reaction was carried out at temperatures of 2, 22 and 50°C, respectively. The results indicate that moderate changes in room temperature did not have a significant effect on the final fluorescence intensity.

i. Effect of the change of order of addition of reagents on the fluorescence intensity.

A reaction between propionic anhydride and $BF_3.Et_2O$ took place when these two reagents were allowed to be in contact with each other for a short time before the solvent and chloroform standard were added. Normally these reagents were added separately to the solvent. The formation of a bright yellow color was observed when the solvent was added. The effect of this side reaction in reducing the fluorescence intensity of the indolopyrone at 480 mμ is shown in Figure 12 (curve B). This curve resulted when the reaction was allowed to proceed for 3 minutes before solvent and IAA were added.

j. Indoles tested and the specificity of the reaction.

As a test of specificity the new technique was used to determine the occurrence of any reaction with the following indoles: tryptophan, tryptamine, 5-hydroxytryptamine (serotonin), IAA, 5-HIAA, indole-3-lactic acid, indole-3-propionic acid, indoleacetamide and indolealdehyde. Fluorescence reactions were given only with IAA and 5-HIAA. A slow reaction

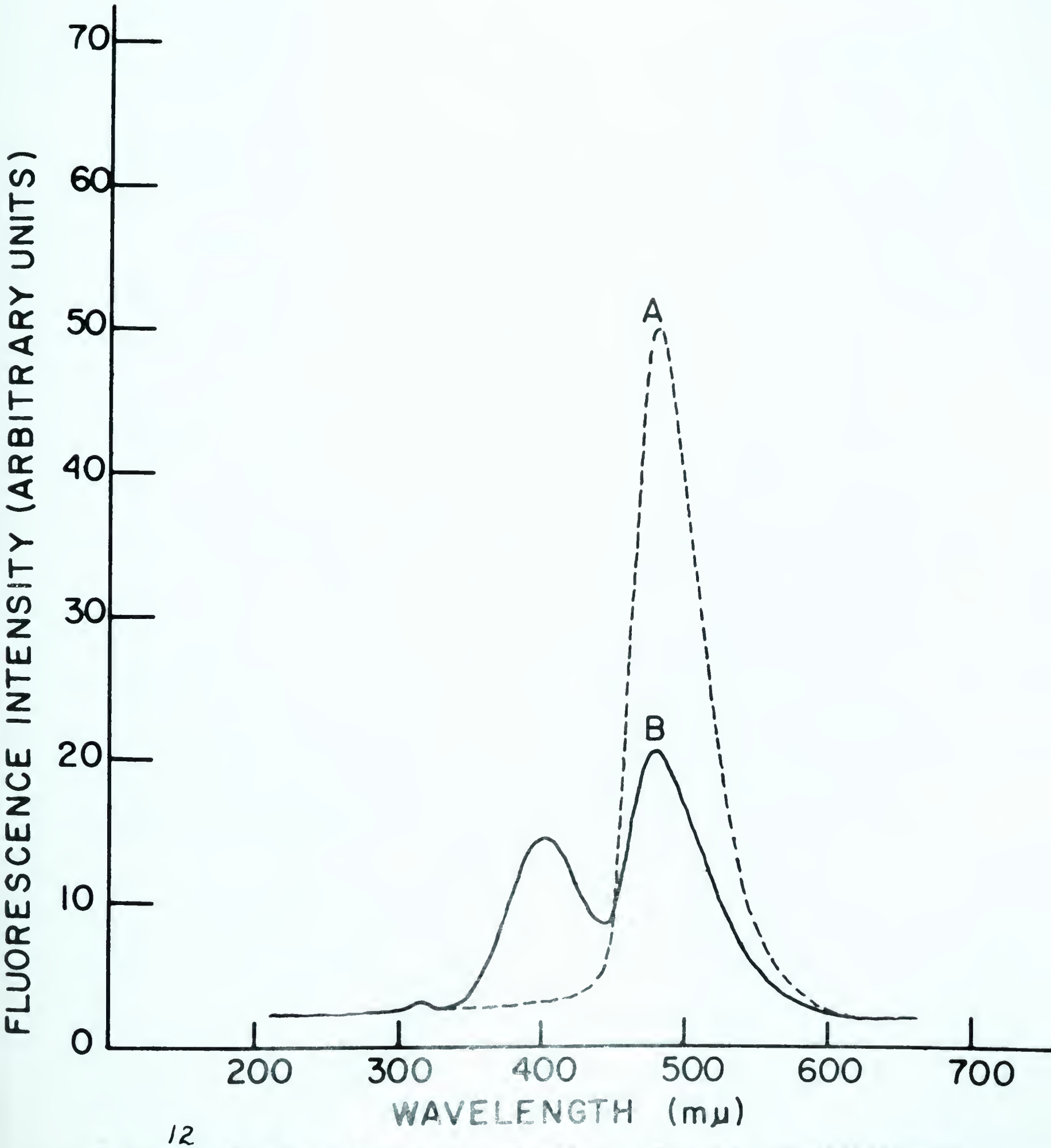


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Fig. ~~16.~~ Spectral study of the effect of order of addition of reagents. Curve A is a fluorescence scan of the mixture when the reaction is performed in the regular manner. Curve B is a fluorescence scan of the mixture when the order of addition of reagents is altered.

occurred with indoleacetamide, but this is not a normal constituent of urine (21). IAA and 5-HIAA are easily separated by extraction with $CHCl_3$.

Further evidence of the specificity of the method was derived from a list of over 50 normal urine constituents that fluoresced. This was supplied by the American Instrument Co., Inc., Silver Spring, Md., in their Supplemental Data Sheet #1. The activation and fluorescence maxima values of the indoles listed in Table II were taken from the Data Sheet. It should be noted that there is no combination of λA and λF that coincides with the wavelengths used for the proposed fluorimetric technique. This is also the case for all of the substances listed in the Supplemental Data Sheet, except for vitamin A which has activation and fluorescence maxima at 325 and 470 mμ, respectively. However, other parameters, such as relative sensitivity, concentration and solubility, are involved. Vitamin A has a fluorescence intensity approximately 100 times less than that of the indolopyrone reaction product. No problems were encountered in the measurement of IAA in urine of patients taking Vitamin A.

TABLE II

Activation and fluorescence maxima of several indoles

Compound	λ A	λ F
Tryptophan	285	365
Tryptamine	290	360
5-HIAA	300	355
Indole	280	355
Serotonin	295	340
IAA	285	345
Indolopyrone derivative of IAA	316	480

3. STUDIES USING PURIFIED INDOLOPYRONE

a. The ultraviolet absorption studies of the pure indolopyrone in ethanol.

Plieninger and Müller (19) reported that the indolopyrone reaction product of IAA in ethanol exhibited three distinct absorption bands. A purified sample of indolopyrone was obtained from Dr. Plieninger and the absorption spectrum was determined (Figure 13). The absorption peaks obtained

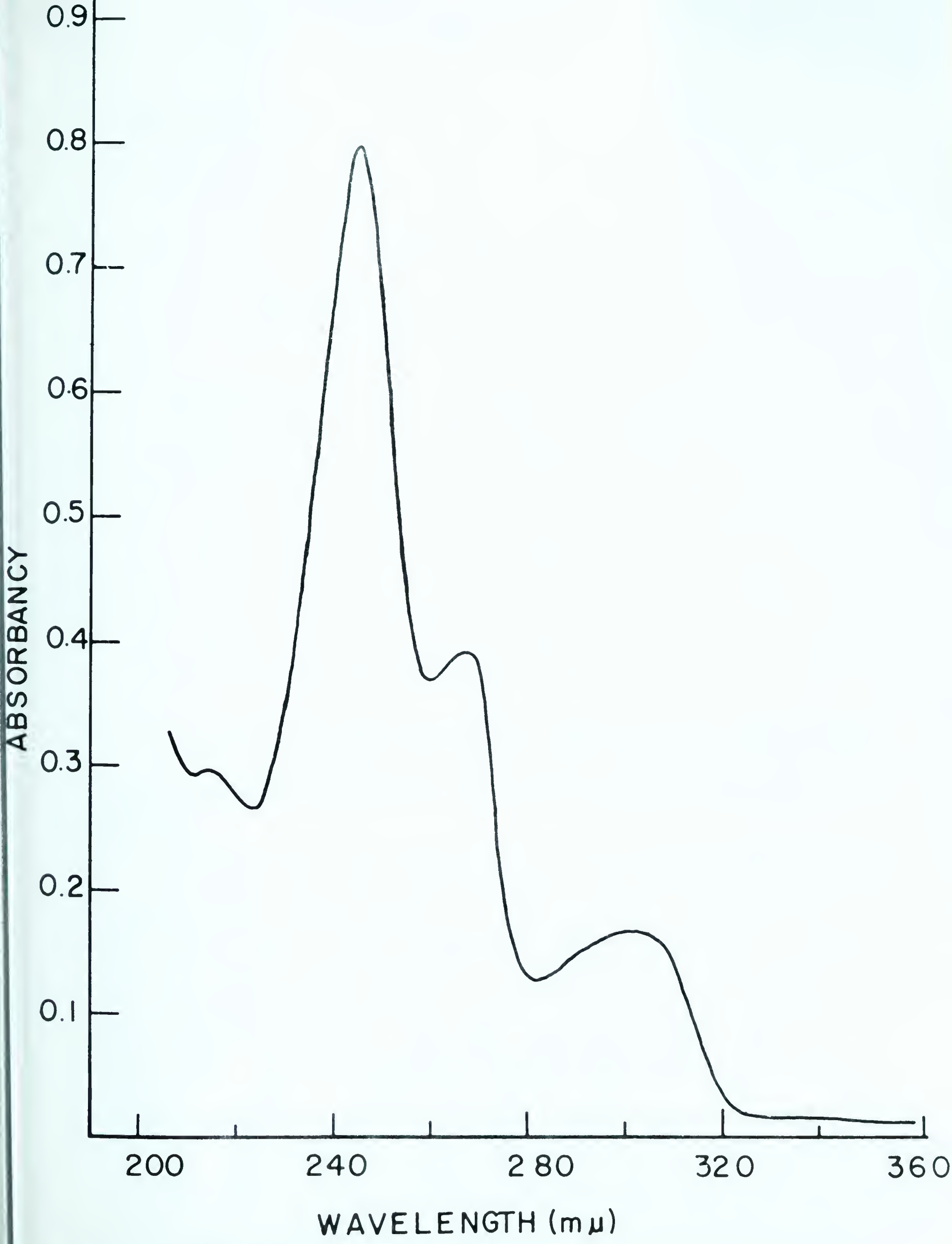

Fig. 13. Ultraviolet absorption spectrum of pure indolopyrone in 95% ethanol.

in 95% ethanol are listed in Table III along with the values published by Plieninger and Müller (19).

TABLE III

Absorption peaks (mμ) of the Indolopyrone in 95% ethanol

Values from Figure 13	Published by Plieninger and Müller (19)
214	-
244	245
267	270
302	305

b. Activation and fluorescence spectra of the pure indolopyrone derivative in the reaction medium and the effect of reagents.

Studies of the pure indolopyrone in chloroform-ether solvent yielded much lower fluorescence readings than produced by an equivalent amount of IAA carried through the reaction. Investigation revealed that indolopyrone fluorescence is markedly increased in the presence of $Prop_2O$ and $Bf_3.Et_2O$ (Figure 14). Possibly this increased fluorescence is due to a complex formed between reagents and indolopyrone. Evidence for this comes from a study of the activation scans shown in Figure 15. The indolopyrone-reagents spectrum with its sharp peak at 316 mμ markedly differs from the spectrum of the indolopyrone in the solvent without

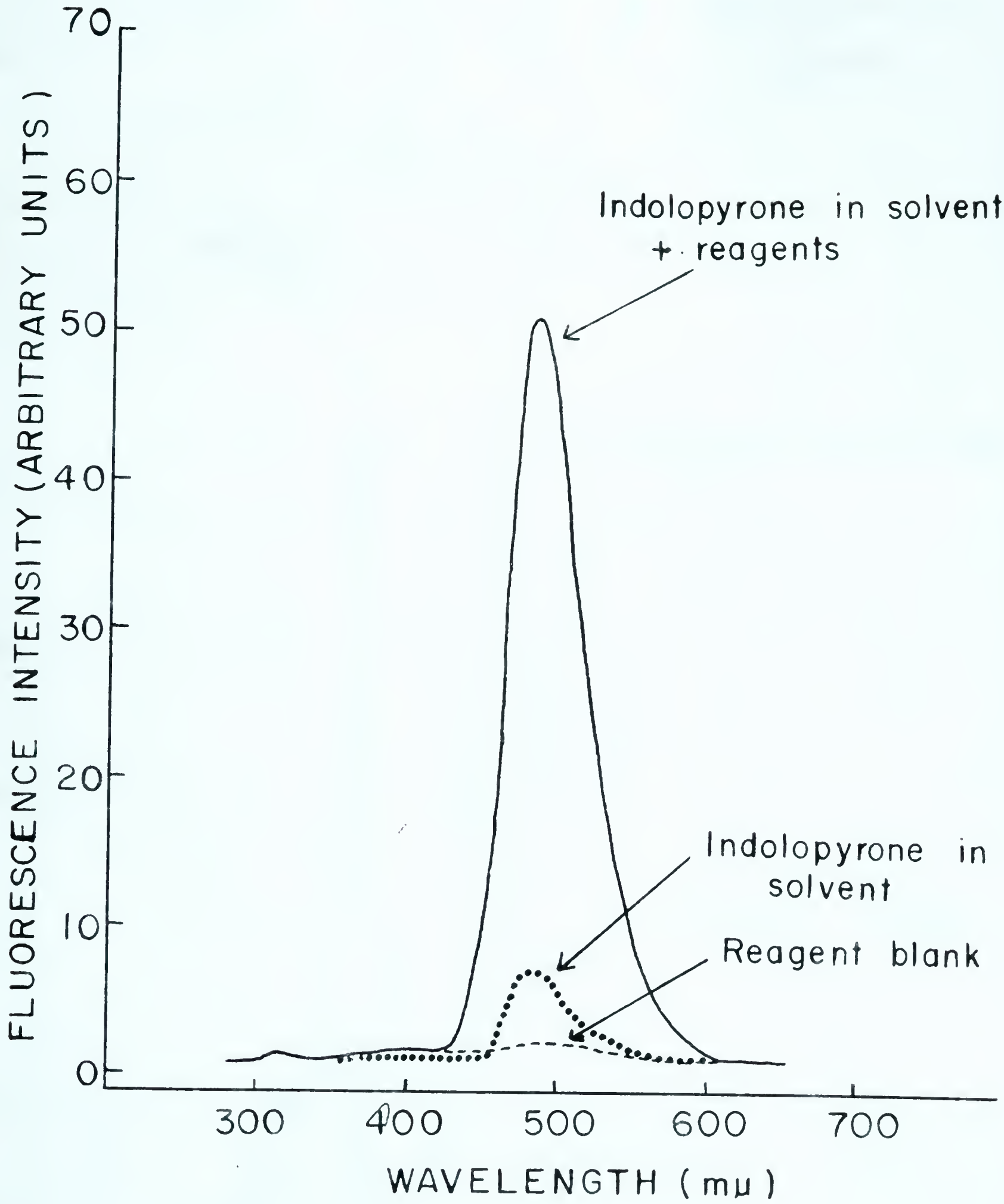

Fig. ~~15~~ 14. Fluorescence curves at λ_A = 316 mu showing the effect of reagents on the pure indolopyrone derivative.

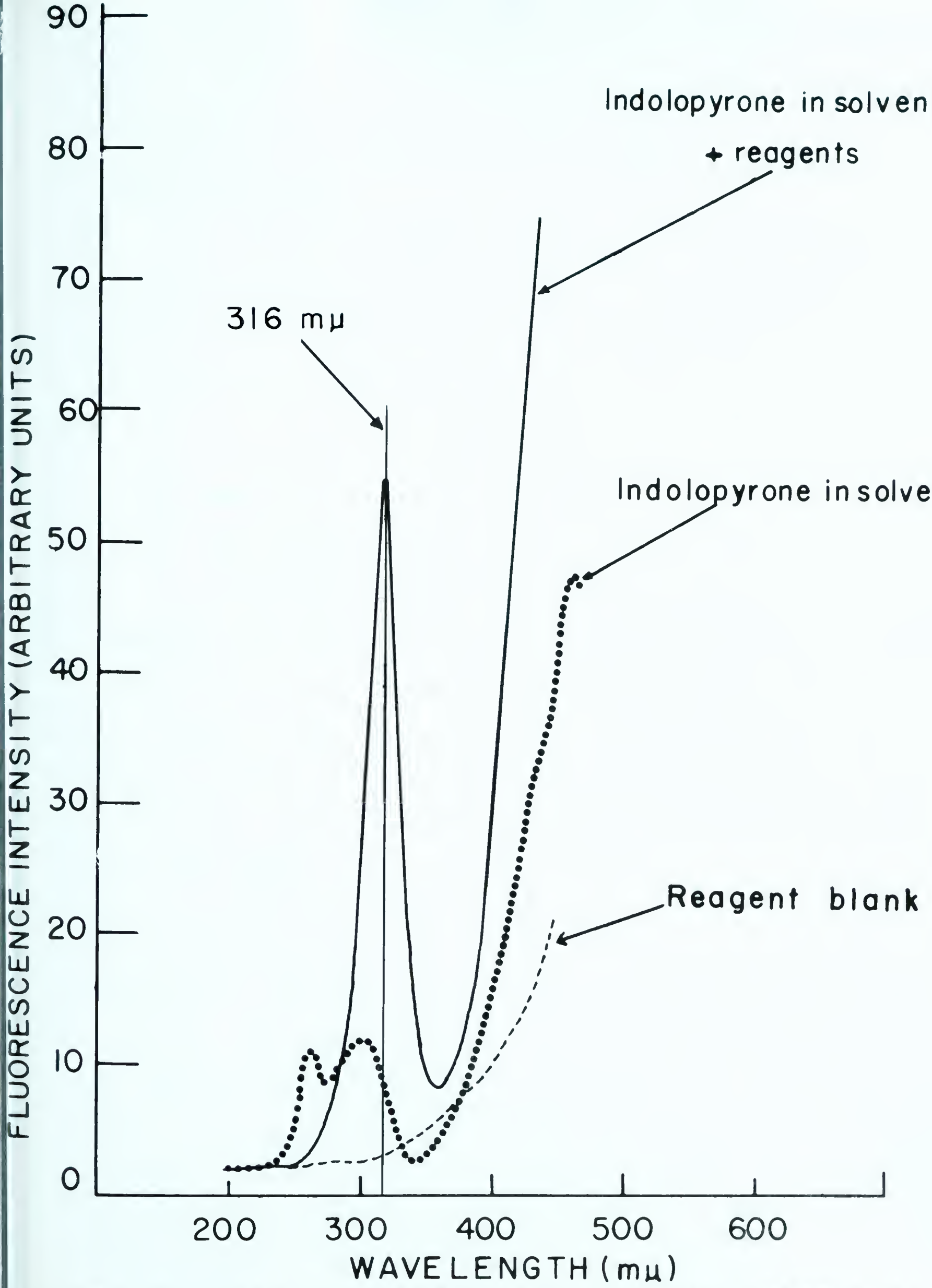

Fig. 15. Activation curves at $\lambda_f = 480$ mu showing the effect of reagents on the pure indolopyrone derivative.

the reagents, thus indicating the presence of a different fluorescent species.

This study also helped to explain why a much lower fluorescence intensity was obtained when, in earlier work, the reaction product was transferred to an aqueous medium. $BF_3.Et_2O$ and $Prop_2O$ are decomposed by water.

4. THE REACTION AS AN ANALYTICAL METHOD

a. The standard curve.

The relationship between IAA concentration and fluorescence intensity was studied. As seen from Figure 16, the relationship between fluorescence response and concentration of IAA in $CHCl_3$ is not quite linear in the range of zero to 1.0 μg./ml. This lack of linearity was also demonstrated in the range of zero to 0.25 μg./ml. of IAA when this range was expanded by increasing instrument sensitivity. It was concluded that this deviation from linearity must be inherent in the system and was probably due either to quenching of the fluorescence or possibly self-absorption. The possibility of self-absorption at first seemed unlikely. The term is generally used to express the effect of an excessive concentration of the fluorescent species so that there was a significant absorption of the exciting light (24). Since the concentration of indolopyrone produced in the reaction was small it was felt that another explanation must be sought. Referring again to activation spectrum of indolopyrone in the reaction mixture (Figure 3), it can be seen that a form of self-

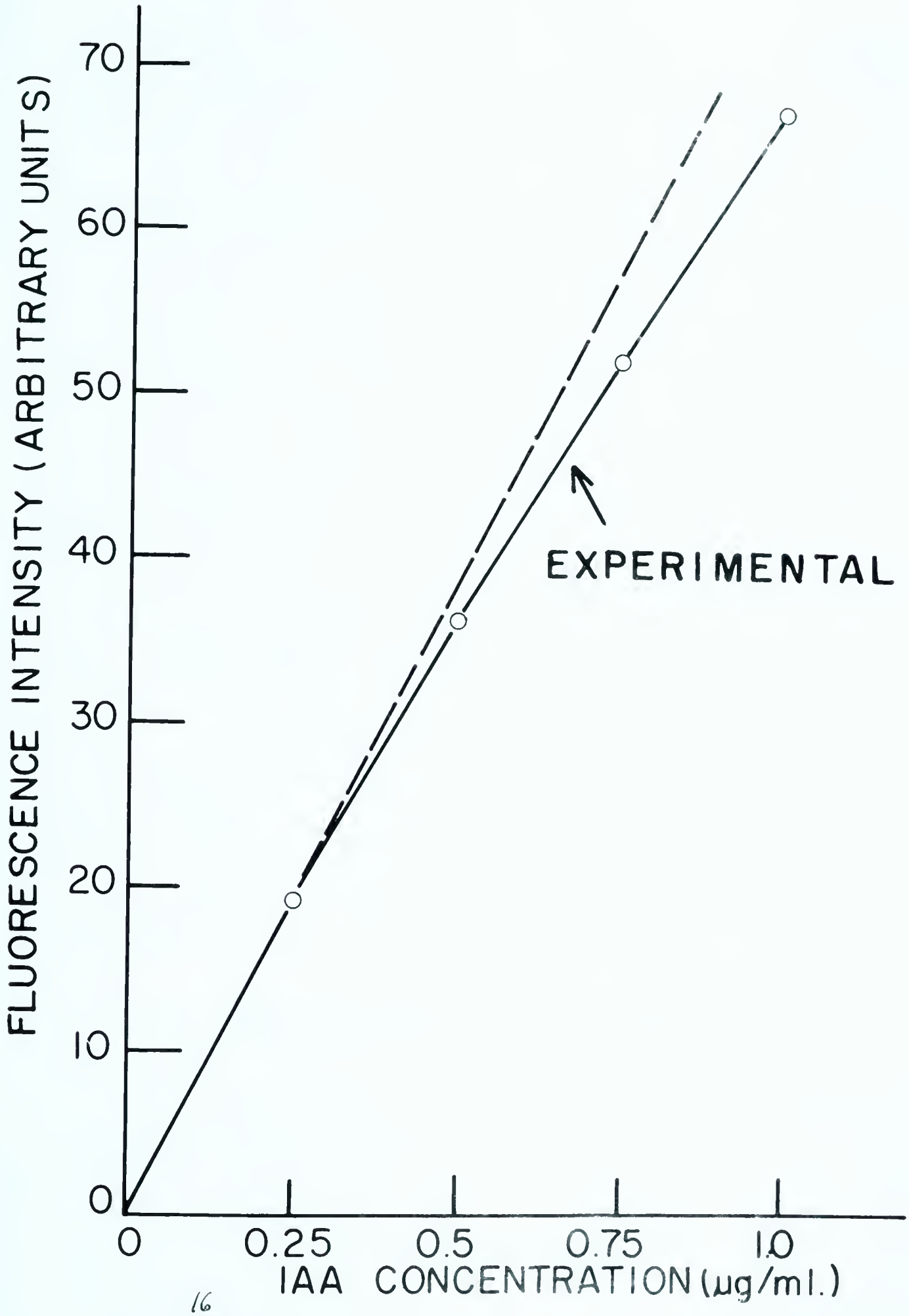

Fig. ~~17~~ 16. Variation of fluorescence intensity with concentration of IAA. Quantities of a standard solution of IAA in $CHCl_3$ were measured out to give a concentration range of zero to 1.00 ug./ml. of final reaction mixture. The fluorimetric reaction was performed directly on the $CHCl_3$ solutions of IAA. A straight line was drawn through the origin and the first experimental point (dashed line).

absorption by indolopyrone may still apply, although the situation was somewhat different than that described by Parker and Rees in their review on fluorescence spectrometry (24). The third activation peak of indolopyrone at 445 mμ overlaps a large portion of the fluorescence band. This may cause the absorption of a small part of the fluorescence produced by the same fluorescent solute. A further clarification of this situation was obtained from further studies of the absorption characteristics of the reaction mixtures. Figure 17 shows the absorption curves of the reagent blank (solid line) and the fluorescent reaction mixture with increasing concentrations of IAA. There was increasing absorption in the 315 mμ region, as well as in the 445 mμ area, as more indolopyrone was formed. This latter absorption band partly overlapped the fluorescence band at 480 mμ, and it was evident that there would be increasing absorption with increasing concentration of the fluorescent product.

b. The sensitivity of the method.

The ultimate sensitivity of the method was determined according to the definition given by the American Instrument Co., Inc., suppliers of the Aminco-Bowman Spectrophotofluorometer, in one of their supplemental data sheets. They defined ultimate sensitivity as the concentration in micrograms per milliliter which will produce a useful (10% of full scale) deflection of the instrument at maximum sensitivity, with optimum arrangement of slits and correct choice of photomultiplier tube. On this basis, as little as 20 nanograms of IAA per ml. can be detected when converted to indolopyrone. This

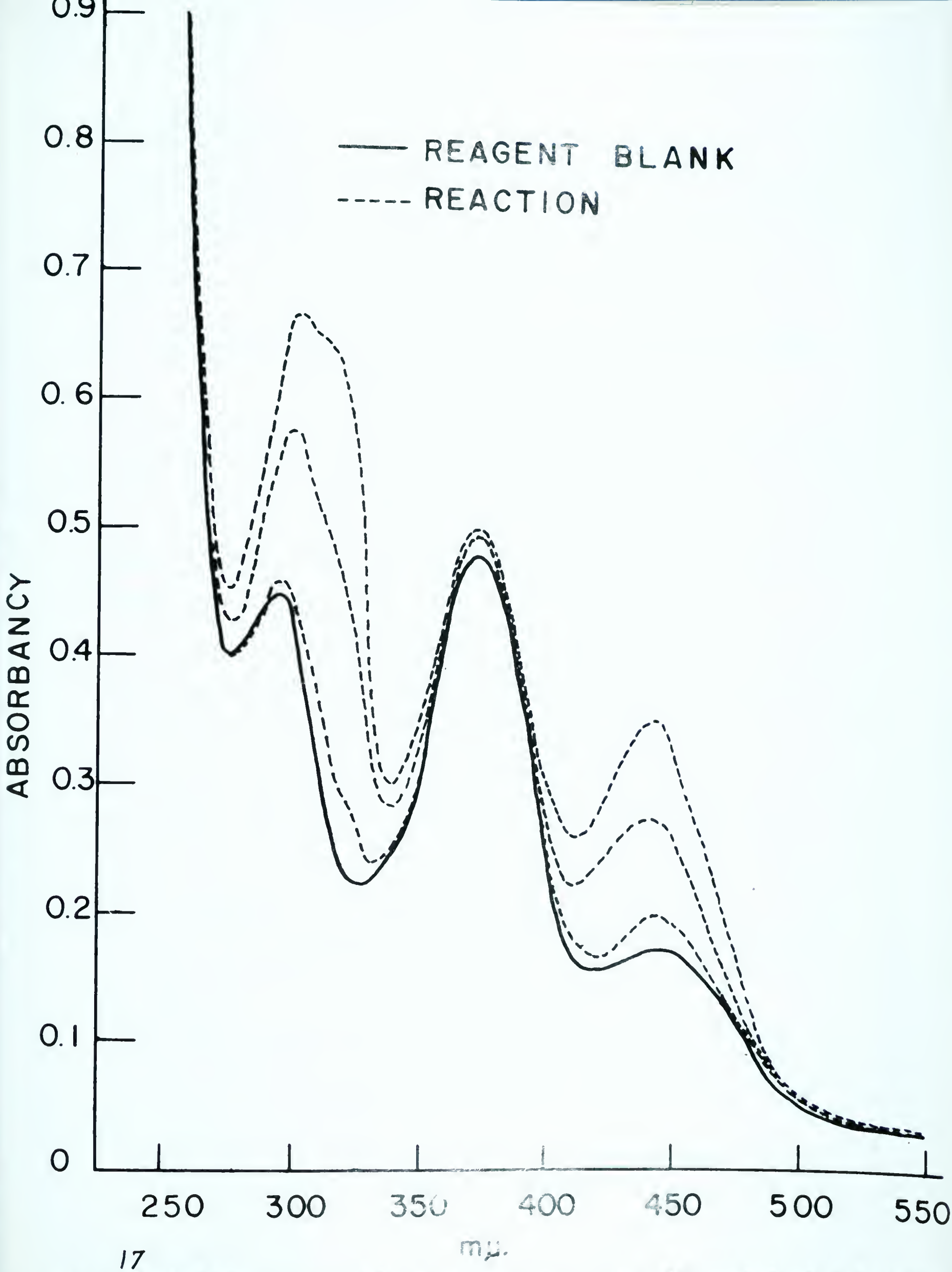

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Fig. ~~16~~. Absorption curves of reagent blank and fluorescence reaction mixture with increasing concentrations of TAA (1,3, and 5 ug./ml.).

is 1/10th the quantity necessary for the measurement of IAA by its native fluorescence and 1/100th of the quantity necessary for the colorimetric analysis by the method of Weissbach, et al. (1).

III. APPLICATION OF THE METHOD TO THE ESTIMATION OF INDOLE-3-ACETIC ACID IN URINE

All previous work dealt with the fluorescence measurement of pure IAA in non-aqueous systems. The measurement of IAA in urine involves extraction from an aqueous medium into a non-aqueous solvent, thus the stability of IAA in water must be considered.

It has been reported by Ruhland (18) that IAA is unstable in acid and stable in alkali. Brauner (23,24) in his work on the photolysis of IAA, observed that aqueous solutions were not affected by incandescent light, but that decomposition took place under light from a quartz lamp. This decomposition was inhibited by guaiacol or ascorbic acid. Of further interest was the suggestion in Weissbach's procedure (1) that a "trace" of urine must be added to preserve the stability of aqueous IAA standards.

To check these various points, the effects of light, pH and urine constituents upon the stability of IAA in solution were studied as follows.

Ten micrograms of IAA in 4 ml. of H_2O were made 1 N with respect to HCl in a series of tubes. One half of these tubes were placed in the dark and the remainder placed under fluorescent lighting. Individual tubes in each series were extracted at 15-minute intervals with 10 ml. $CHCl_3$ and the fluorimetric

reaction was carried out on an aliquot of the $CHCl_3$ extract. Other tubes, to which 0.05 ml. of urine had been added, were treated similarly. The results, shown in Table IV, indicated that IAA is unstable in acid aqueous medium. Light has no effect on stability.

TABLE IV

The effect of incubation in 1 N HCl on the stability of IAA in aqueous solution

Sample	Incubation period (minutes)	Fluorescence intensity (a.u.)	
		In light	In absence of light
1	0	20.0	-
2	15	13.5	14.5
3	30	11.0	12.0
4	45	9.5	10.5
5	60	9.5	10.0
6 + urine	0	41.5	-
7 + urine	30	42.5	42.5

However, IAA in the solution containing a trace of urine constituents was found to to stable. This was not due to extraneous fluorescence from urine constituents, since, when 0.05 ml. of urine was added to water, extracted and read in the same manner, no measurable fluorescence was observed.

Further experiments showed that as little as 0.01 ml. of urine possessed the same "preservative" capacity. When samples 1 and 6 are compared, it is apparent that approximately 50% of the IAA in sample 1 is destroyed immediately when the solution is made 1 N with respect to HCl.

In the next experiment, the reported protective action of ascorbic acid in acid pH was investigated. $CHCl_3$ extracts of 4 ml. of aqueous IAA solutions in 1 N HCl containing 0.5% ascorbic acid, when carried through the usual fluorescence reaction, failed to give fluorescence readings as high as those obtained with extracts of solutions containing 0.01 ml. of urine.

In the work cited above, double distilled water had been used to make up IAA standards. At this time some difficulties were being encountered elsewhere in the laboratory in measuring traces of hemoglobin in feces with o-tolidine in that false positives were occurring. It was realized that this might be due to a high concentration of Cl_2 in the double distilled H_2O used to dilute the stool specimen. Ortho-tolidine is also a sensitive reagent for chlorine. It was considered possible that the oxidizing action of Cl_2 in acid might be the cause of the observed destruction of IAA.

To investigate this possiblility, varying amounts of both de-ionized and distilled water were added to 1 ml. IAA standard (prepared in de-ionized water). The results are shown in Table V.

As in the experiment described in Table IV, all samples were acidified with HCl and extracted with $CHCl_3$, except that the extractions were made immediately. As can be seen, there

TABLE V

The effect of double distilled water on fluorescence intensity

Sample No.	ml. of standard IAA (10 μg./ml. H_2O)	Distilled H_2O (ml.)	De-ionized H_2O (ml.)	Urine (ml.)	Fluorescence reading (a.u.)
1	1	0	3	-	42.5
2	1	1	2	-	35.5
3	1	2	1	-	29.5
4	1	3	0	-	29.5
* 5	1	0	3	0.01	43.0
** 6	1	0	3	0.01	1.0

* Sample No. 5 contained a trace of urine for comparison.

** Sample No. 6 is a reagent control.

is a progressive decrease in fluorescence intensity in the presence of increasing amounts of distilled water. It was also found that the addition of distilled water that had been heated and then cooled yielded a fluoresence value identical to that when de-ionized water was used. Thus the substance present causing the diminishing of IAA fluorescence was volatile and in all probability was chlorine.

Regarding the stability in alkali, it was found that IAA standards in 0.1 N NaOH were completely stable for at least seven months, as observed by absorption and fluorescence measurements.

Indole-3-acetic acid occurs in urine in both free and conjugated forms (5,13,21). The initial urinary studies concerned the extraction and measurement of the free form. The problem of the measurement of total IAA is discussed later (page 57).

1. ACTIVATION AND FLUORESCENCE SPECTRA OF THE REACTION PERFORMED ON URINARY EXTRACTS

Four milliliters of normal urine were extracted with 10 ml. $CHCl_3$ and the Plieninger-Müller reaction was carried out on the $CHCl_3$ extract (Figure 18). Note that the wavelength of maximum fluorescence of the reaction product is identical to that obtained with pure IAA solutions as seen in Figure 3. This was regarded as satisfactory evidence that IAA was present and that the method might be adaptable to urine.

Examination of the fluorescence curve of the indolopyrone reaction product of IAA from a $CHCl_3$ extract of urine (Figure 18) shows a background fluorescence greater than that of the reagent blank. This is particularly evident in the 400 mμ region. This implies that other substances with fluorescence properties are extractable with $CHCl_3$. This may contribute to the fluorescence reading of IAA at 480 mμ. In order to measure this spurious background fluorescence at 480 mμ, the following procedure was adopted.

A reagent blank consists of reagents minus IAA. Preparation of a sample blank, in this case urine, is not as simple a procedure. The problem was solved in the following manner. Propionic anhydride was replaced by propionic acid in the reaction mixture. This prevented the formation of the fluorescent indolopyrone derivative, but enabled the measurement of the background fluorescence. The urine blank thus consisted of 7.6 ml. $CHCl_3$, 2.0 ml. ethyl ether, 0.1 ml. propionic acid, 0.3 ml. $BF_3.Et_2O$.

Next, known amounts of IAA were added to urine, extracted and measured fluorimetrically. Only 30% of the IAA was recovered when compared to unextracted standards. The apparent low recoveries were either due to incomplete extraction of IAA and/or fluorescence quenching by extraneous material.

2. EXTRACTION EFFICIENCY STUDIES

The extraction efficiency was investigated using aqueous standards. Figure 19 shows the variation of extraction efficiency with pH (solid line). Five milliliters of

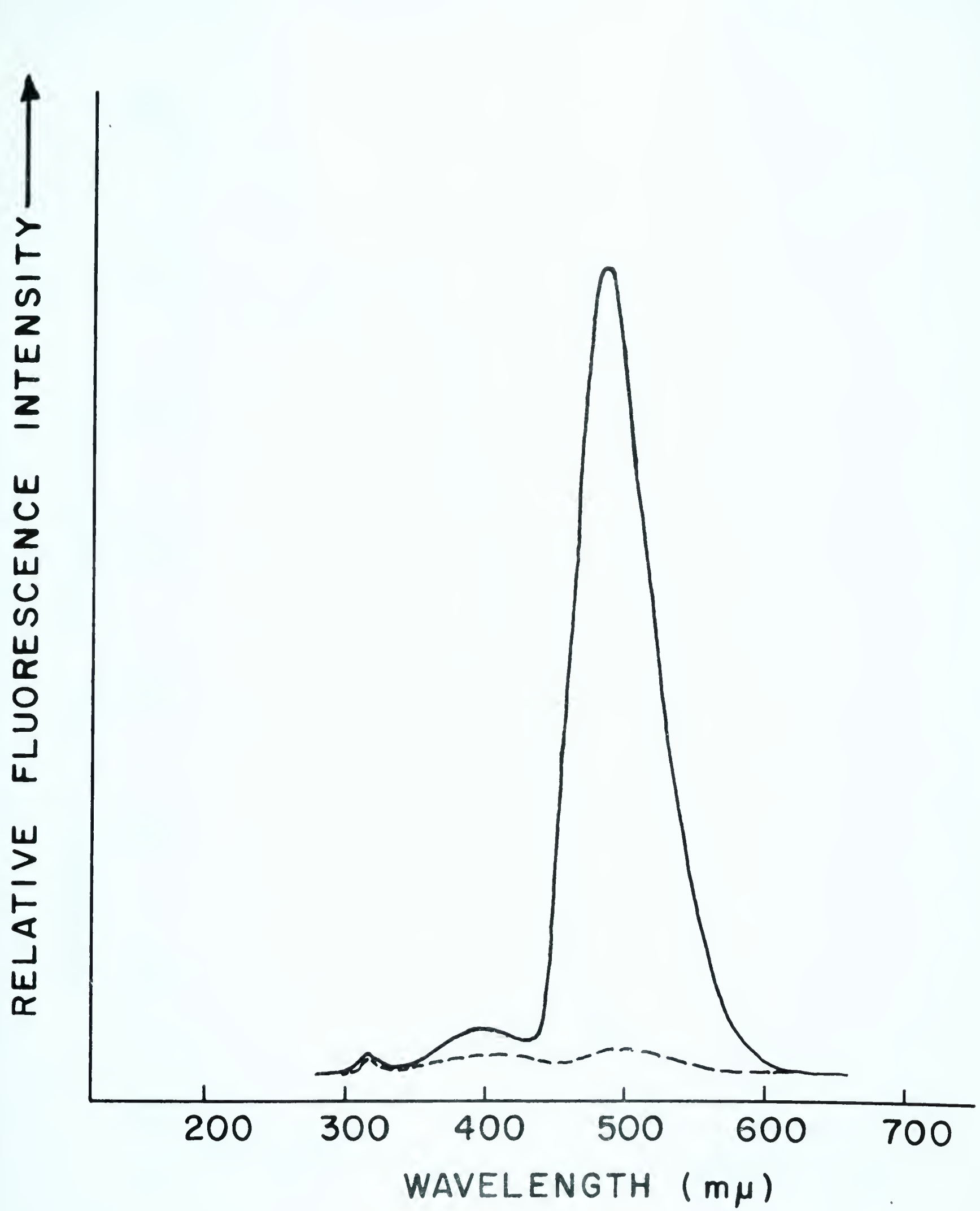

Fig. 18. Fluorescence scan of indolopyrone reaction product formed when IAA is determined in a $CHCl_3$ extract of 4 ml. of normal urine (solid line). Activation wavelength = 316 mu. Fluorescence maximum = 480 mu. The fluorescence scan of the reagent blank is also shown (dashed line).

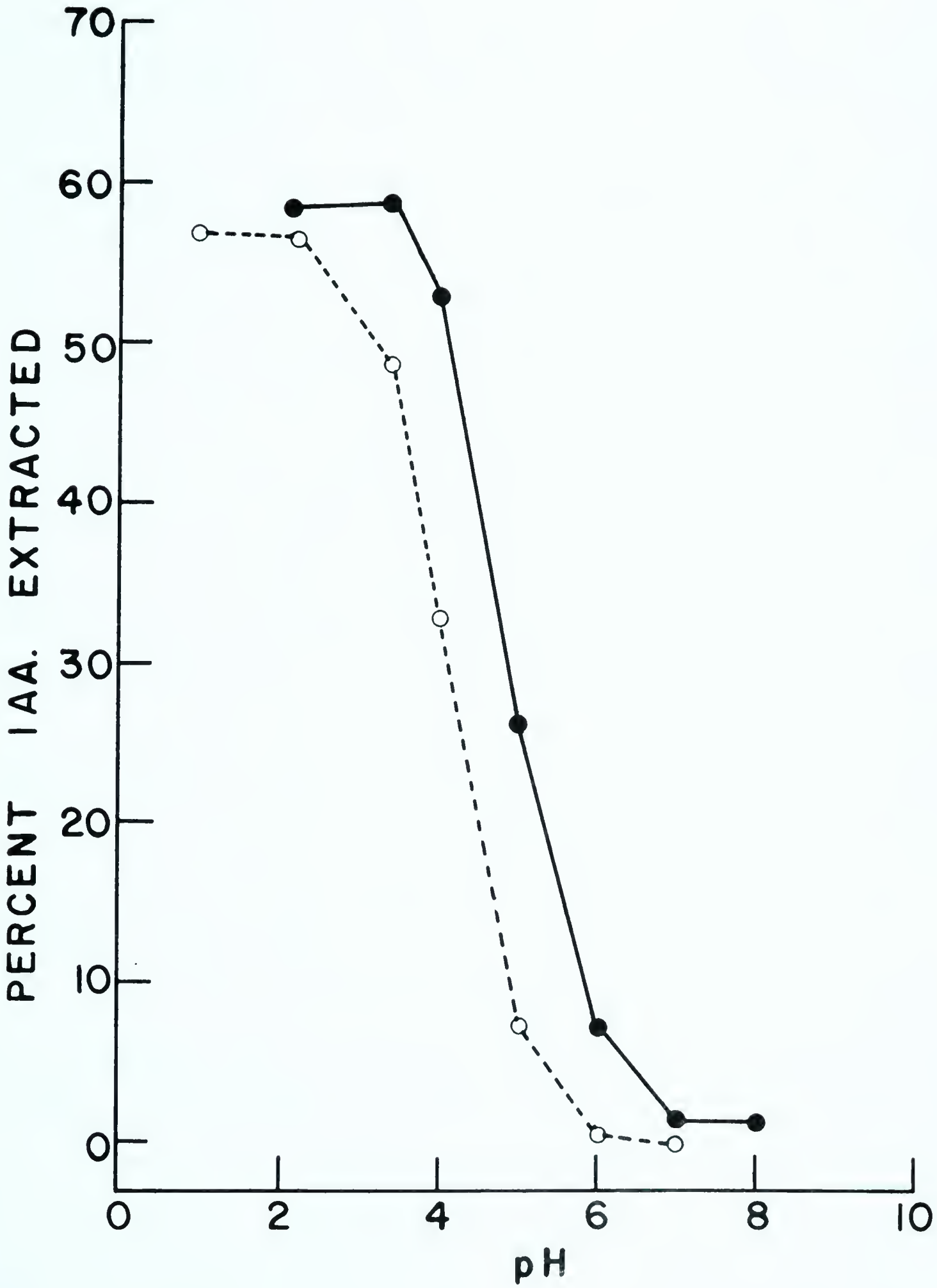

Fig. 19. Extraction efficiency as a function of pH (solid line). Five milliliters of aqueous IAA were extracted with 10 ml. $CHCl_3$ and the absorbancy of IAA in $CHCl_3$ was measured at 278 mu. A theoretical curve is shown (dashed line) which was calculated using the pKa of IAA in water and the partition coefficient of IAA when the volume ratio of water to $CHCl_3$ is 1 to 2.

aqueous IAA (20 μg./ml.) were extracted with 10 ml. $CHCl_3$ and the concentration of IAA in $CHCl_3$ was measured by reading the absorbance at 278 mμ. At a pH of 2.7, the maximum amount of IAA extracted was 59%. When a 4-ml. volume of IAA standard was extracted instead of a 5-ml. volume, the extraction efficiency was about 65%. Under identical extraction conditions, it was stated in the previous section that only 30% recovery was obtained when IAA was added to a 4-ml. volume of urine. This indicated that a significant amount of quenching was also a factor in the fluorimetric technique. Included in Figure 19 is a theoretical curve (dashed line) which was calculated using 3.8 as the pKa of IAA in water (13) and the partition coefficient of 0.75. This coefficient was determined with the aqueous phase adjusted to 1 N with HCl and a volume ratio of water to $CHCl_3$ of 1 to 2. Two equilibria are involved: the salt to acid ratio at a given pH in the aqueous media, and the partition between the aqueous and $CHCl_3$ phases of the unionized form of IAA.

Figure 20 illustrates the extraction efficiency of 5 ml. of aqueous IAA (20 μg./ml.) standard with different volumes of $CHCl_3$ (solid line). Included in Figure 20 is a theoretical curve (dashed line) which was determined in a similar manner as for Figure 19. It can be seen from Figure 20 that recoveries of approximately 77% were obtained at a volume ratio of 1 to 4. When 2 ml. of the standard IAA solution were extracted with 10 ml. $CHCl_3$, recoveries were 80%. However, extractions of 2 ml. of urine with 10 ml. of $CHCl_3$ still yielded lower and variable recoveries based on fluorescence analysis. Prevention

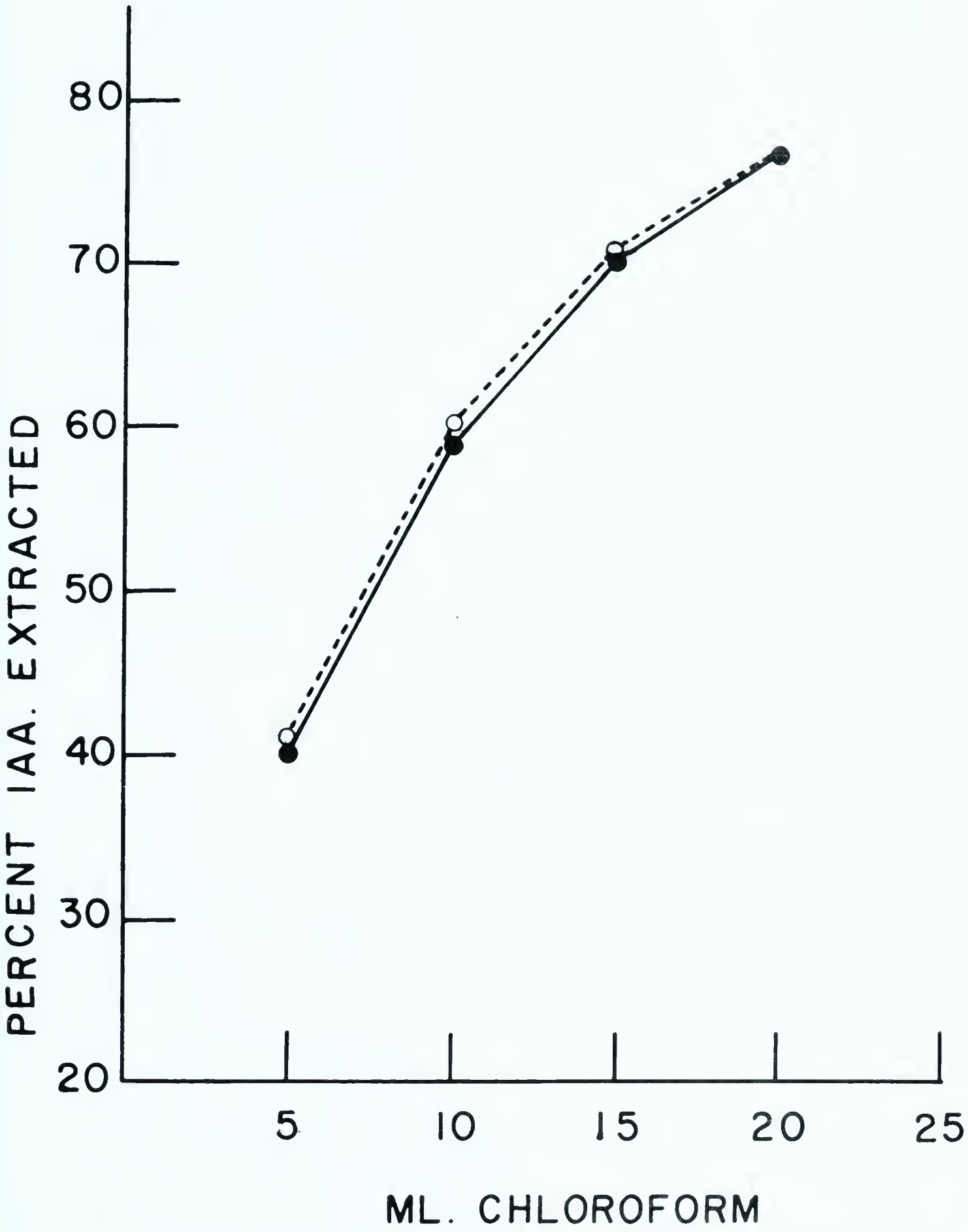

Fig. 20. Extraction efficiency as a function of volume of $CHCl_3$. Five milliliters of aqueous IAA were extracted at pH 1.0 (solid line). A theoretical curve is shown (dashed line) which was calculated using the pKa of IAA in water and the partition coefficients for each $CHCl_3$-water mixture.

of emulsion formation by using 0.5 ml. of urine plus 1.5 ml. of water did not increase the recovery of added standards. Thus this difficulty was not due to lower extraction efficiency and must be attributed to quenching of the fluorescence reaction by other urine constituents extracted by $CHCl_3$.

Evidence of the pronounced quenching effect is given in Table VI. Amounts of urine from 0.1 to 1.0 ml. were made up to 2 ml. volume with water. These were extracted with 10 ml. of $CHCl_3$ and the IAA , as indolopyrone was measured fluorimetrically. A pronounced non-linear relationship between the volume of sample extracted and the amount of IAA recovered was observed.

TABLE VI

The quenching effect of urine

ml. urine	ml. H_2O	Fluorescence (a.u.)
0.1	1.9	2.7
0.2	1.8	5.3
0.4	1.6	9.9
0.6	1.4	13.0
0.8	1.2	17.3
1.0	1.0	19.8

3. THE REMOVAL OF INTERFERING SUBSTANCES FROM URINE

An investigation into the possibility of overcoming the fluorescence quenching effects of extraneous substances extracted from urine was carried out.

Several types of column materials were tested for their ability to separate IAA from interfering materials in the urine. It was hoped that this could be accomplished by adsorption of IAA onto the column followed by removal of interfering materials by washing and subsequent elution of IAA, or by the adsorption of interfering materials with the removal of IAA in the column effluent.

a. Charcoal.

Charcoal columns were first tested. Preliminary work with activated charcoal showed that it had the property of decolorizing the urine as well as adsorbing the IAA. However, the IAA could not be eluted with NaOH or HCl. Charcoal was used by Marko and Reynolds (25) in their isolation of indican, which also has an indole nucleus. They deactivated charcoal with stearic acid and eluted the adsorbed indican with phenol reagent. It was decided to apply this technique to the isolation of IAA from urine.

Both adsorbing and decolorizing charcoal* were deactivated with stearic acid, as described by Marko and Reynolds

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* Fisher Scientific Co., Ltd.

(25) and used for the preparation of columns 8 mm. in diameter and 10 to 15 mm. high. One to two ml. samples of urine with and without added IAA as well as pure aqueous standard were run through separate columns and then washed with two 1-ml. portions of de-ionized water. Elution of adsorbed IAA with three 2-ml. portions of 0.7 M phenol in H_2O was incomplete and the work on the charcoal column was abandoned.

b. Ion exchange resins.

The cellulose exchange resins were tested first. These included DEAE-C, TEAE-C, ECTEOLA, and CM-C. IAA was not adsorbed on these columns, nor were the quenching substances removed.

Strong anionic Dowex resins 1 and 2 in the chloride form showed a strong affinity for IAA. Either of these resins completely removed IAA from urine or aqueous standards. However, elutions with 1 N and 2 N acid or alkali were unsuccessful. The column dimensions were 8 mm. inside diameter and approximately 8 to 10 mm. in length. Volumes of sample applied varied from 2 to 4 ml. In general, indoles are very difficult to remove from ion exchange resins (26).

Dowex-3, a weak anionic exchanger in the chloride form, was next studied. When 4 ml. of IAA solution (10 µg./ml.) were added to a column about 7 to 8 mm. in height and 8 mm. inside diameter, approximately 80% of the IAA was recovered when eluted with 20 ml. of 0.1 N NaOH. However, the quenching substances in urine were still present in the IAA eluate fraction when urine was passed through the column.

Dowex-50 in the hydrogen form gave poor recoveries when 2 ml. of urine at a pH of approximately 5.0 was run through and washed with an equal volume of water. The effluent retained the fluorescence quenching properties.

c. Alumina.

Three types of chromatographic alumina were used: acid (pH 4), neutral and alkaline (pH 10). Columns 18-20 mm. in length were set up as described in previous work. When 5 ml. of pure aqueous solutions of IAA were passed through the columns, 89% of the IAA was adsorbed at pH 4.0, 11% at pH 7.0 and zero adsorption at pH 10.0. Using the same length of columns, 1 to 2 ml. samples of acidified urine (to 1 N with HCl) were passed through a pH 4 alumina column and compared with another sample to which a known amount of IAA was added and passed through another identical column. The volumes of water required to elute the adsorbed IAA also eluted the quenching substances and separation was not achieved. Work with alumina was then suspended.

d. Florisil.

Florisil is a magnesium silicate preparation available from the Floridin Co., Tallahassee, Florida, and is commonly used for the removal of chromogenic material from urine.

Columns were made 18 mm. in length and 8 mm. in diameter. Two ml. of IAA standard were applied to the columns. The results showed that incomplete recoveries were obtained when the columns were washed with an equal volume of water. Further washing was not carried out, as it caused colored urinary

constituents to pass through when urine samples were employed. A neutral pH was used.

This work was repeated at various pH levels. Six columns were set up and samples made up as shown in Table VII were applied. Under acid conditions, the columns retained the colored constituents of urine, whereas under alkaline conditions, these constituents passed through. However, no significant differences were obtained for the recovery of IAA added to the urine as shown in the final column of Table VII. This indicated that the quenching substances in urine were colorless constituents. The recovery from urine was approximately 80 -90% when compared with aqueous standards passed through identical columns. An example is given below using a different sample of urine.

1 ml. urine + 1 ml. H_2O ..... 11.5 a.u.

1 ml. urine + 1 ml. IAA standard (10 μg.) .... 24.0 a.u.

1 ml. H_2O + 1 ml. IAA standard (10 μg.)..... 16.5 a.u.

$$\frac{24.0 - 11.5}{16.5} \times 100 = 81.8\% \text{ recovery}$$

Only 1-ml. urine samples were employed in this work. It is recalled that in earlier work approximately 30% recovery of IAA from urine was obtained. Initially, 4 ml. of urine were extracted, and very low recoveries resulted. An example is given.

4 ml. urine ..... 17.0 a.u.

4 ml. urine + 40 μg. IAA ..... 31.1 a.u.

4 ml. H_2O + 40 μg. IAA ..... 44.0 a.u.

TABLE VII

IAA recoveries from Florisil columns at various pH values

Column*	Sample	pH	Fluorescence (a.u.)	Relative IAA recovery (a.u.)
1	1 ml. urine + 1 ml. H_2O	5	2.0	-
2	" " + 1 ml. standard**	5	28.3	26.3
3	" " + 1 ml. H_2O	7	2.5	-
4	" " + 1 ml. standard	7	27.3	24.8
5	" " + 1 ml. H_2O	9	3.5	-
6	" " + 1 ml. standard	9	29.5	26.0

* The columns were eluted with 2 ml. water.

** A standard IAA solution of 20 μg./ml. was used.

$$\frac{31.1 - 17}{44} = 32.1\% \text{ recovery}$$

Florisil failed to remove the fluorescence quenching substances from urine and was not considered further.

e. Removal of drug metabolite.

The $CHCl_3$ extracts of urine samples from patients on drug therapy for malsbsorption often exhibited abnormal fluorescence patterns. In some cases these peaks overlapped the indolopyrone fluorescence peak. As these patients are administered a great variety of drugs, it was difficult to determine which drug or metabolite was responsible for the abnormal fluorescence peak. A typical spectrum is shown in Figure 21. Note the fluorescence peak at 400 mμ which is absent in extracts of normal urine. None of the column materials tested removed these interfering substances. However, this problem was overcome by determining the fluorescence at 480 mμ of a urine blank, prepared as described previously. To determine IAA, the fluorescence value of the blank at 480 mμ was subtracted from the fluorescence of the indolopyrone reaction, the difference being the concentration of IAA.

4. THE INTERNAL STANDARD

The problem of quenching was overcome by the addition of a known amount of IAA to the urine specimen as an internal standard. The ability of an internal standard to compensate for quenching is demonstrated in Table VIII. In the 4th column the gradual decrease in recovery of the added 5 μg. IAA can be seen with the increasing concentration of the urine. The uniform values of the IAA content of the urine

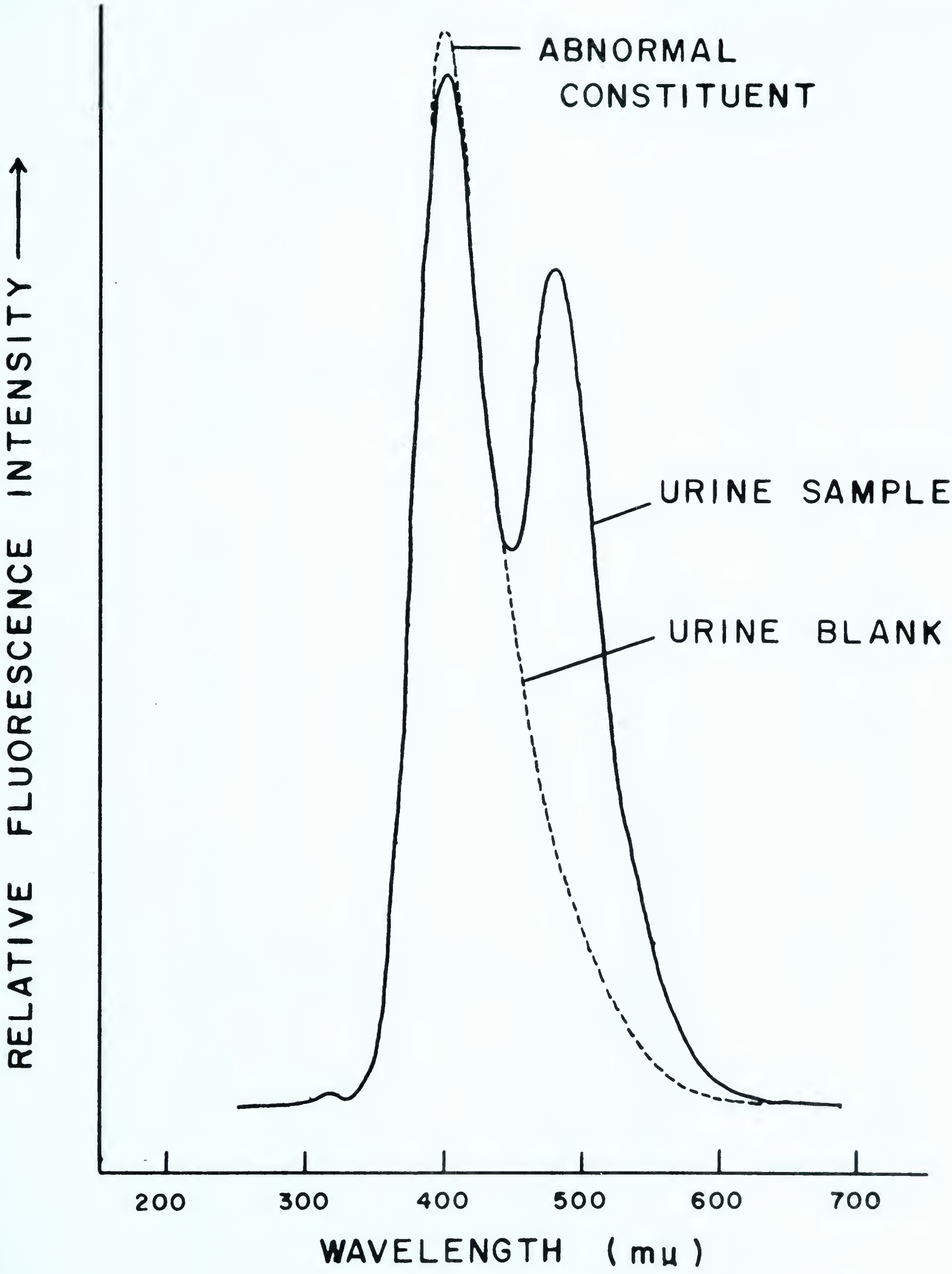


Fig. ~~22.~~ 21 Fluorescence spectra of urine sample and blank of a patient under treatment with drugs. λ_A = 316 mu.

TABLE VIII

IAA recoveries from various dilutions of urine in a 2-ml. volume

ml. urine per 2 ml. volume	Total fluorescence with 5 μg. IAA added (a.u.)	Fluorescence without IAA added (a.u.)	Fluorescence difference (added IAA)	IAA/ml. urine* (μg.)
0.1	48.2	2.7	45.5	3.0
0.2	48.7	5.3	43.4	3.1
0.4	49.7	9.9	39.8	3.1
0.6	51.4	13.0	37.4	3.1
0.8	52.5	17.3	35.2	3.1
1.0	50.0	19.8	30.2	3.3

* The calculation for the recoveries of IAA were performed as follows:

$$\frac{\text{Fluorescence intensity of sample}}{\text{Fluorescence intensity of sample + internal standard minus fluorescence intensity of sample}} \text{ X IAA added (5.0 μg.) X } \frac{1}{\text{volume of urine}} = \text{μg. IAA/ml. urine.}$$

For 0.1 ml. of sample this is calculated as follows:

$$\frac{2.7}{48.2 - 2.7} \text{ X } 5 \text{ X } \frac{1}{0.1} = 3.0 \text{ μg. IAA/ml. urine.}$$

obtained by correcting for quenching and extraction efficiency by use of an internal standard is shown in the last column.

Next was considered the amount of IAA to be added to urine as an internal standard by the standard addition method (27). If a straight line relationship exists between concentration and instrument response, then concentration of the unknown can be calculated using the Beer-Lambert equation. However, a slight deviation from linearity is involved (see Figure 16). In analytical methods where non-linearity exists (failure of Beer's Law), unknown concentrations are determined by bracketing the unknown reading with two external standards whose concentration is just slightly less and slightly more than that of the unknown. This minimizes the error due to the non-linear relationship between instrument reading and concentration. This approach cannot be used when an internal standard is required.

One method to overcome this problem is to correct the observed value by use of a factor that will linearize the curvature. Another approach is to employ a series of standard additions of different quantities. The resulting net values are plotted against the concentration of the standard solutions which were added to the unknown. The line is extrapolated to the ordinate at which point is indicated the value of the unknown sample. This approach is not practical for routine analysis of large numbers of specimens.

Tests were performed to determine the error involved in the present method if a single standard were employed without using a correction factor. It is evident that when one uses

the Beer-Lambert relationship for the calculation of results based on a non-linear function, accuracy varies directly with the closeness of the standard and unknown readings. Accordingly, if one chose a concentration of internal standard that was low enough so that when added to urine it would result in a reading not much greater than that of the urine unknown alone, but great enough to be commensurate with adequate instrument precision, then the error would be minimal.

Two internal standards of 10 and 20 μg. IAA were added per ml. of urine sample. The fluorescence reading of the 10 μg. standard, when doubled, gave, on the average, a value approximately 3% higher than that of the 20μg. standard. A 5 μg. internal standard gave deviations from linearity that were less than 3% and also gave a sufficiently large incremental increase to the reading of the unknown so that the instrument could detect the increase with precision. Thus this concentration of internal standard was used for analysis of urine and no correction factor was employed.

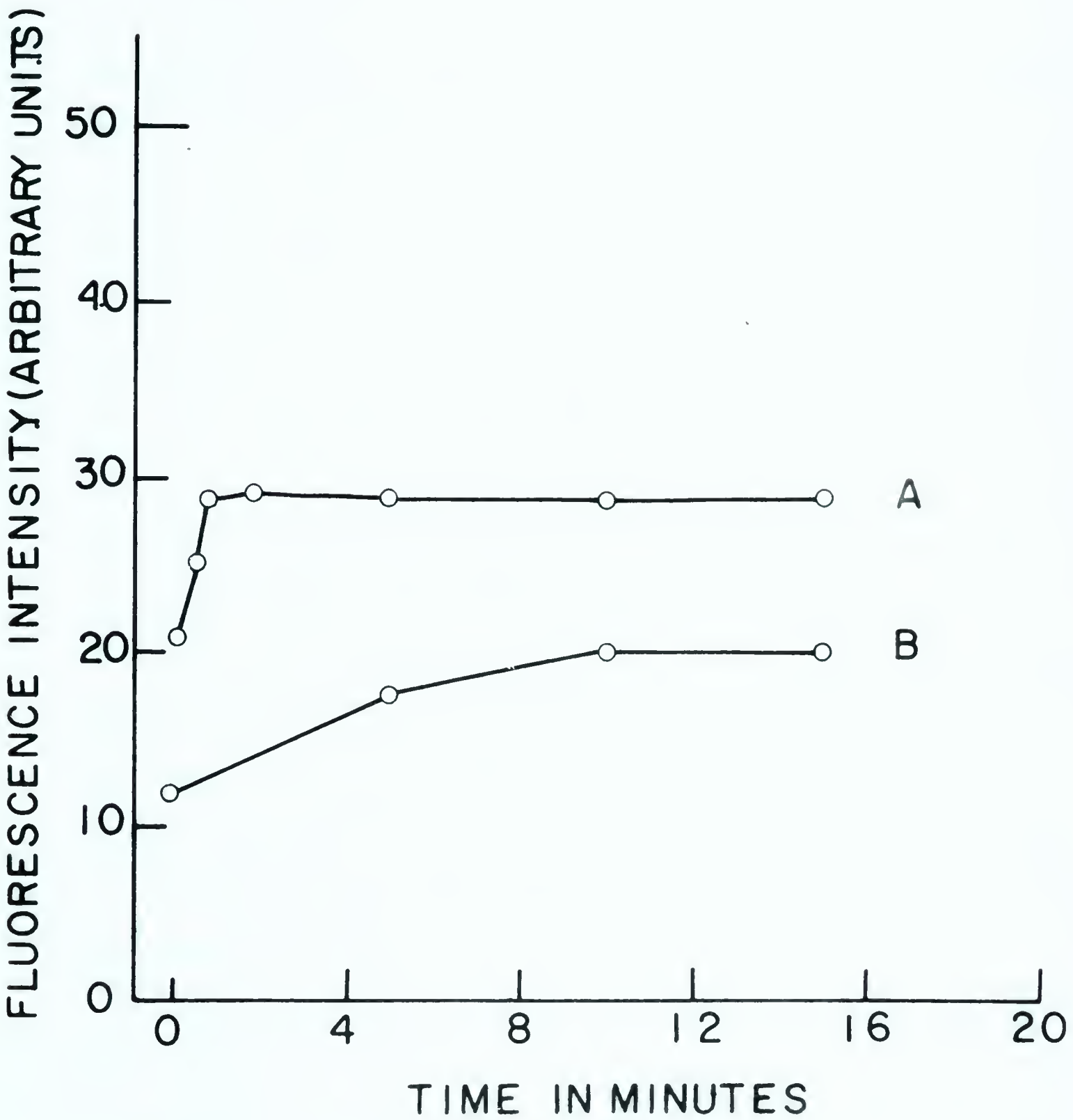
5. A STUDY OF HYDROLYSIS FOR "TOTAL" IAA

Weissbach, *et al*. (1) attempted alkaline hydrolysis of urine since indoles are stable in alkaline solution. They found that hydrolysis of urine at 100°C in 1 N NaOH resulted in a marked increase in the apparent IAA present as determined by colorimetric measurement. They showed that, besides

measuring IAA, they were measuring other indole acids that had been liberated by alkaline hydrolysis. When they tried acid hydrolysis this also resulted in an increase of the IAA value due only to increased IAA, based on chromatographic studies of the acid hydrolysate.

Although Weissbach, et al. (1) adopted acid hydrolysis for the estimation of total urinary IAA, it was felt that both acid and alkaline hydrolysis should be investigated using the present more specific technique. Accordingly, two 4-ml. aliquots of a sample of urine were adjusted to 1 N with respect to HCl and NaOH, respectively, and hydrolyzed at 100°C for 15 minutes. Following the acidification of the alkaline sample, both samples were extracted with $CHCl_3$ and the fluorescence intensity was evaluated. The alkaline hydrolyzed sample yielded a significantly higher fluorescence value than the acid hydrolyzed sample. The same finding occurred when the alkaline hydrolysis was carried out at room temperature.

Hydrolysis time curves were run on aliquots of a urine sample adjusted to both acid and alkaline conditions as described above. Alkaline hydrolysis was stopped by the addition of acid. The results are shown graphically in Figure 22. As was expected, alkaline hydrolysis yielded significantly higher values (curve A). Also, alkaline hydrolysis at room temperature liberated more IAA in a much shorter time (less than 2 minutes) than acid hydrolysis at 100°C (curve B). The acid hydrolysis time curve compared closely with a similar curve run by Weissbach, et



22.
Fig. ~~21~~. Hydrolysis time curves of a sample of urine. At the end of the alloted times, the samples were extracted with 10 ml. $CHCl_3$ and the fluorescence reaction performed. Curve A represents hydrolysis in 1N NaOH at room temperature. Curve B represents hydrolysis in 1N HCl at 100° C.

al. (1). It was found that urine adjusted to 0.5N with NaOH gave the same results as hydrolysis in 1N NaOH.

It was thought that the lower values obtained by acid hydrolysis were due to partial destruction of IAA because of the lower stability of IAA in acid media. This was born out by the work of Weissbach, et al. (1) who carried out a hydrolysis time curve of urine with the acidity adjusted to 6N with respect to HCl. No IAA was recovered. To check for any instability of IAA in 1N HCl, the follwing was carried out. Two samples of equal concentration of IAA in water were prepared and made 1N with HCl. To each was added a trace of urine as a precaution against any other influences than the acid conditions. One sample remained at room temperature and the other was placed in boiling water for 15 minutes. Both samples were then extracted with $CHCl_3$ and indolopyrone fluorescence was determined. Surprisingly, both samples yielded identical results. It was therefore thought that no destruction of IAA occurred due to acid conditions of 1N with respect to HCl.

The higher total IAA obtained with alkaline hydrolysis, when compared to acid hydrolysis (Figure 21) of urine, remains unexplained. A possible explanation for this disparity is derived from the work of Edwards and associates (14). They stated that indoleacetylglycine in urine was partly destroyed during acid hydrolysis. It seemed therefore reasonable to suppose that the lower values obtained on acid hydrolysis when compared to alkaline hydrolysis could be due to destruction of some conjugated forms of IAA present in urine. In

the above experiment, where IAA was not destroyed when hydrolyzed at 100°C in 1N acid solution, only the unconjugated form was present.

The very short hydrolysis time required in an alkaline medium was in agreement with the work of Jepson (21). He found that the glucuronide, which is the major urinary conjugate, was extremely rapidly converted to glucuronic acid and IAA by alkali. The effect of acid conditions was not evaluated. Another IAA conjugate discussed by Jepson (21) is indoleacetylglutamine. This conjugate is not as readily hydrolyzed as the glucuronide. Thus, possibly the true total IAA is not being obtained with mild alkaline hydrolysis.

Nevertheless, it was decided that alkaline hydrolysis should be adopted in the procedure and that the amount of IAA measured following alkaline hydrolysis be designated "total" IAA.

6. PROCEDURE FOR THE QUANTITATIVE ESTIMATION OF IAA IN URINE

PRINCIPLE: See the description of the Plieninger-Müller reaction.

APPARATUS AND MATERIALS:

1. Aminco-Bowman Spectrophotofluorometer. American Instrument Co. Inc., Silver Spring, Maryland.
2. 40-ml. glass-stoppered round bottom centrifuge tubes.
3. Test tubes, 5/8" x 4".

REAGENTS:

1. $CHCl_3$, reagent grade, water saturated. $CHCl_3$ is thoroughly

shaken with an equal volume of de-ionized water and is allowed to stand overnight in the dark or in a brown bottle. The water is then aspirated off and the lower $CHCl_3$ layer used. This procedure tends to remove impurities as well as destroy any phosgene present.

2. Ethyl ether, anhydrous reagent grade. The peroxides are removed from ether (20) by passing it through a column of activated aluminum oxide (acidic). The water content of ether is adjusted to 0.15% by adding de-ionized water. Shake well. Refrigeration will reduce peroxide formation.
3. Boron trifluoride ethyl ether, purified. Fisher Scientific Co. Cat. No. 4272.
4. Propionic anhydride, highest purity. Fisher Scientific Co. Cat. No. 1291.
5. Propionic acid, reagent grade. Fisher Scientific Co. Cat. No. A-258.
6. Sodium hydroxide solution, 0.1 N.
7. Sodium hydroxide solution, 12 N.
8. Hydrochloric acid, 12 N.
9. Indole-3-acetic acid. Nutritional Biochemicals Corporation, Cleveland, Ohio.
10. Indole-3-acetic acid stock standard, 100 µg./ml. in 0.1N NaOH. This is stable for at least 7 months in the refrigerator.
11. Indole-3-acetic acid working standard, 5 µg./ml. Prepare fresh each day from the stock standard by diluting 1 in 20.

PROCEDURE: Prepare two glass-stoppered 40-ml. centrifuge tubes for extraction as follows: To each add 0.5 ml. of urine. To one labeled "a-b" add 1.5 ml. de-ionized water, and to the other labeled "c" add 1.0 ml. de-ionized water and 0.5 ml. of working IAA standard (2.5 μg.).

FREE IAA: To each tube add 0.18 ml. 12N HCl and 10 ml. $CHCl_3$. Shake the tubes for 5 minutes. After centrifugation, discard the upper aqueous phase and transfer a 3.8 ml. portion of $CHCl_3$ extract from the tube labeled "a-b" to each of two test tubes labeled "a" and "b". Transfer a 3.8 ml. portion of $CHCl_3$ layer from the second centrifuge tube labeled "c" to a third test tube similarly labeled. The tubes are then treated with propionic anhydride and $BF_3.Et_2O$ as described under "Total" IAA.

TOTAL IAA: Another two glass-stoppered 40-ml. centrifuge tubes are prepared for extraction. To each add 0.5 ml. of urine. To one labeled "a-b" add 1.5 ml. de-ionized water and to the other labeled "c" add 1.0 ml. de-ionized water and 0.5 ml. of working standard.

To each tube add 0.09 ml. 12 N NaOH mix, and allow to stand for 5 minutes. Then add 0.27 ml. of 12 N HCl to each tube which will neutralize the NaOH present and render the solution 1 N with respect to HCl. To each tube add 10.0 ml. $CHCl_3$ and shake for 5 minutes. After centrifugation, the upper aqueous phase is discarded and 3.8 ml. portions of $CHCl_3$ extract are transferred to test tubes as described for "free" IAA.

To test tubes marked "a" add 0.05 ml. of propionic acid. To test tubes marked "b" and "c" add 0.05 ml. of propionic anhydride. Immediately to all 3 tubes add 1.0 ml. of ethyl ether and 0.15 ml. of $BF_3.Et_2O$. The fluorescence intensity in arbitrary units (a.u.) is read at 30 minutes in the spectrophotofluorometer with an activation wavelength set at 316 mμ and a fluorescence wavelength setting of 480 mμ.

CALCULATIONS:

$$\frac{\text{Fluorescence intensity of sample (b)} - \text{Fluorescence intensity of sample blank (a)}}{\text{Fluorescence intensity of sample + standard (c)} - \text{Fluorescence intensity of sample (b)}} \times \text{IAA added (2.5 μg.)}$$

$$\times \frac{1}{\text{volume of urine}} = \text{IAA concentration in μg./ml. urine.}$$

OR $$\frac{b - a}{c - b} \times 2.5 \times \frac{1}{0.5} = \text{μg. IAA/ml. urine.}$$

OR $$\frac{b - a}{c - b} \times 5.0 = \text{μg. IAA/ml. urine.}$$

On the basis of a 24-hour volume:

$$\frac{\text{μg. IAA/ml. urine} \times \text{urine volume in ml.}}{1000} = \text{mg. IAA/24 hrs.}$$

On the basis of creatinine excretion:

$$\frac{\text{mg. IAA/24 hrs.}}{\text{g. creatinine/24 hrs.}} = \text{mg. IAA/g. creatinine.}$$

Example:

Fluorescence readings obtained:

Sample blank (a) ..... 1.6 a.u.

Sample (b) ..... 21.0 a.u.

Sample + standard (c) ..... 38.5 a.u.

Formula:

$$\frac{b - a}{c - b} \times 5.0 = \mu g.\ IAA/ml.\ urine.$$

Substituting:

$$\frac{21.0 - 1.6}{38.5 - 21.0} \times 5.0 = \frac{19.4}{17.5} \times 5.0$$

$$= 5.6\ \mu g.\ IAA/ml.\ urine.$$

At this time it would be useful to present a brief description of the colorimetric and fluorimetric method of Weissbach, et al., for comparison and later reference:

IAA IN URINE:- COLORIMETRIC

1. 4.0 ml. of urine are acidified by the addition of 0.36 ml. of 12 N HCl.
2. 10 ml. $CHCl_3$ are added and the tube is shaken for 5 minutes.
3. After centrifugation, 9.0 ml. of the $CHCl_3$ extract are transferred to another 40-ml. centrifuge tube containing 0.6 ml. of 0.5 M phosphate buffer, pH 7.0.
4. The tube is shaken for 5 minutes and 0.4 ml. of the pH 7.0 buffer extract is transferred to a test tube containing 0.4 ml. of 12 N HCl; 1.0 ml. of the xanthydrol reagent

is added, followed 5 minutes later by 0.5 ml. of the bisulfite reagent.

5. The solution is mixed and the pink color is measured within 5 to 10 minutes in a spectrophotometer at 520 mμ.

IAA IN TISSUE:- FLUORIMETRIC

1. A tissue incubation mixture is transferred to a centrifuge tube and the proteins are precipitated.
2. An aliquot portion of the protein-free filtrate is transferred to a 40-ml. centrifuge tube and acidified with HCl.
3. This is extracted with 15 ml. $CHCl_3$ and the aqueous layer discarded.
4. The $CHCl_3$ phase is washed by shaking with 10 ml. of 0.1 N HCl, thus removing any tryptophan that had been extracted with the $CHCl_3$.
5. Ten ml. of the $CHCl_3$ is extracted with phosphate buffer and the fluorescence determined in the buffer in a spectrophotofluorometer (activation wavelength, 285 mμ; fluorescence wavelength, 365 mμ).

The following steps have been eliminated in the new procedure:

(a) Termination at the first $CHCl_3$ extraction stage eliminating a second extraction into phosphate buffer.

(b) Unnecessary to wash the $CHCl_3$ to remove tryptophan. The present fluorimetric reaction is performed directly on the $CHCl_3$ extract.

IV. URINARY EXCRETION OF IAA

1. NORMAL RANGE

In order to establish the normal range for the new fluorimetric technique, a series of 32 determinations was carried out on 24-hour urine specimens of 32 normal individuals. These included 15 females and 17 males of ages ranging from 3 - 79 years. Figure 23, diagram A, shows a histogram of the frequency distribution of total IAA calculated on the basis of mg. IAA/g. creatinine. Also included in this Figure is a histogram of the frequency distribution of the same group calculated on the basis of mg. IAA/24 hours.

As can be seen in Figure 23, the values based on 24-hour excretion were spread over a significantly greater range than those based on creatinine excretion. The reason for this is that creatinine excretion reflects the body weight of the individual. Children would not be expected to excrete as much as adults, but their excretion was comparable when based on creatinine excretion.

The normal range of 2.9 to 12.0 mg. IAA/g. creatinine was obtained at the 95% confidence level (i.e. mean $\pm$ 2 S.D.). The standard deviation was 2.3. Similarly, on the basis of 24-hour excretion, the normal range was 1.5 to 18.4 mg. IAA/24 hours with a standard deviation of 4.25. All samples were analyzed three times. Each value of the triplicate estimation was determined on a different day.

For comparison, the normal range for total IAA excretion was determined by the colorimetric method of Weissbach, et al.

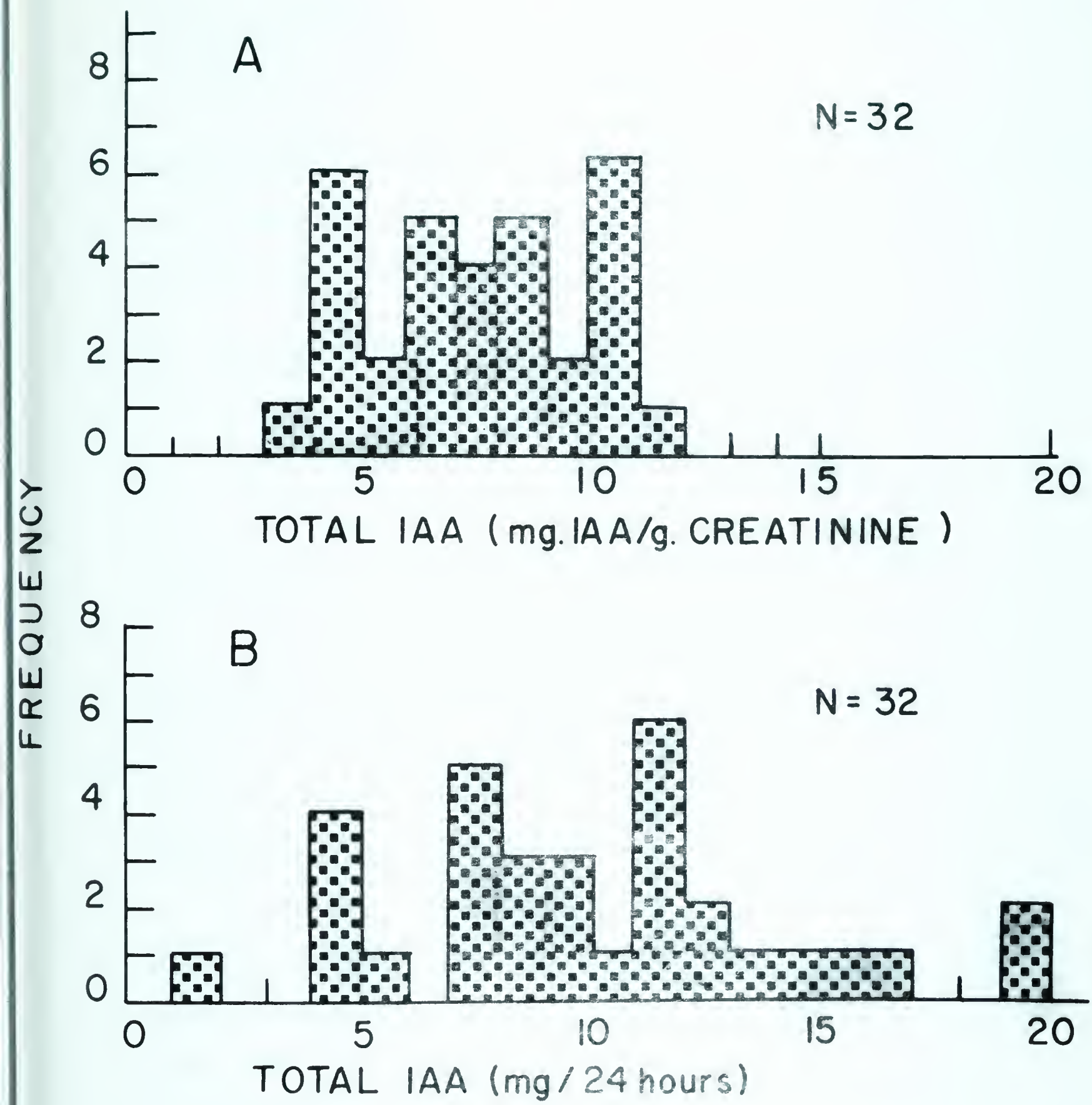


Fig. 23. Frequency distribution of urinary total IAA of normal subjects on the basis of 24-hour urinary volume and on the basis of creatinine excretion.

(1). A normal range of 4.0 to 15.1 mg. IAA/24 hours was obtained at the 95% confidence level.

The mean values for men and women by the present fluorimetric technique were respectively 7.7 and 7.2 mg. total IAA/g. creatinine and showed no significant difference when the t test for comparing averages was carried out.

2. COMPARISON OF THE COLORIMETRIC METHOD OF WEISSBACH, ET AL (1) AND THE PRESENT FLUORIMETRIC TECHNIQUE

Although the figures obtained for the normal range of urinary excretion of IAA by both methods were similar, it was decided to carry out a statistical study of the 2 methods.

Table IX shows that the values for free IAA excretion determined by the fluorimetric method have a standard deviation of 0.26 μg./ml. compared to 0.76 μg./ml. for the colorimetric method. The standard deviations were calculated from differences between the duplicates as described by Youden (28).

As it was suspected that the two methods were yielding different values, the same specimens from 24 normal persons, recorded in Table IX, were analyzed for both the free and total IAA excretion by both methods. The results are given in Table X and are the means of triplicate estimations. The fluorimetric method gave significantly lower values for free IAA excretion, while giving higher values for total IAA excretion. Regarding the significance of the differences, the values of t were 7.95 and 2.64 respectively, both exceeding the critical t values of 2.07 at the 5% level. The lower values obtained for free IAA by the fluorimetric method could

be due to greater specificity of the method. The higher total values are more difficult to explain.

3. NON-SPECIFICITY OF THE COLORIMETRIC TECHNIQUE OF WEISSBACH, ET AL. (1)

Since it was possible that the difference between the methods could be due to the greater specificity of the present fluorimetric technique, it was felt that further useful information in this respect could be derived from a study of the colorimetric reaction.

Spectroscopic readings of the final color produced 1 minute after the addition of the bisulfite reagent (see procedure on page 65) showed no change when read again at 24 hours. This was true for both pure IAA solutions and urine extracts. Apparently bisulfite stops further reaction of IAA with xanthydrol.

The xanthydrol reaction prior to addition of bisulfite was also investigated by reacting xanthydrol with IAA for 5, 10 and 15 minutes before stopping the reaction with bisulfite. Aqueous standards gave the same colorimetric readings at each time interval. However, urine specimens gave increased readings with time. A more detailed investigation of this difference was carried out in the following manner: eight portions of urine from a normal subject were extracted according to the colorimetric procedure. Following the addition of the xanthydrol reagent to each tube, the bisulfite reagent was added to the respective tubes in such a manner that the xanthydrol reaction was allowed to proceed at timed intervals of 1/2, 1, 2, 5, 6, 8, 10 and 15 minutes. The bisulfite reagent

TABLE IX

Free IAA excretion in μg./ml. determined by two methods

Sample	Colorimetric			Fluorimetric		
	Duplicates		Difference^2	Duplicates		Difference^2
1	4.3	2.8	2.25	1.3	1.3	0.00
2	4.6	3.4	1.44	1.8	1.9	0.01
3	6.1	4.7	1.96	3.1	3.2	0.01
4	3.8	5.8	4.00	2.8	3.3	0.25
5	7.8	6.9	0.81	5.5	5.7	0.04
6	5.7	6.3	0.36	3.3	3.6	0.09
7	7.3	6.3	1.00	5.7	6.4	0.49
8	5.7	5.8	0.01	4.4	4.7	0.09
9	5.8	8.8	9.00	4.7	4.5	0.04
10	6.8	8.0	1.44	4.7	5.6	0.81
11	3.3	3.8	0.25	4.1	5.2	1.21
12	4.0	3.5	0.25	3.1	3.3	0.04
13	5.8	5.8	0.00	4.7	5.7	1.00
14	4.2	4.7	0.25	3.7	4.1	0.16
15	3.0	4.2	1.44	2.6	2.4	0.04
16	3.3	2.5	0.64	2.1	2.3	0.04
17	4.5	3.3	1.44	2.4	1.9	0.25
18	1.8	2.3	0.25	2.5	2.4	0.01
19	5.5	6.0	0.25	4.5	5.1	0.36
20	3.7	3.5	0.04	2.9	3.1	0.04
21	2.8	3.2	0.16	2.0	1.9	0.01
22	4.0	3.8	0.04	3.5	3.3	0.04
23	7.5	7.7	0.04	5.4	5.1	0.09
24	6.0	6.1	0.01	4.5	4.6	0.01
Total			27.33			3.13

$S.D. = \sqrt{\frac{1}{2n}(\text{sum of } d^2)}$ Colorimetric, $S.D. = \sqrt{\frac{27.33}{48}} = 0.76$

Fluorimetric, $S.D. = \sqrt{\frac{3.13}{48}} = 0.26$

TABLE X

Comparison of Methods*

Sample	Free (µg./ml.)			Total (µg./ml.)		
	Colorimetric (C)	Fluorimetric (F)	Difference (C-F)	Colorimetric (C)	Fluorimetric (F)	Difference (C-F)
1	3.4	1.2	2.2	4.3	2.8	1.5
2	3.9	1.9	2.0	5.9	4.9	1.0
3	5.4	3.4	2.0	7.0	6.2	0.8
4	5.1	3.0	2.1	11.8	13.9	-2.1
5	7.6	5.6	2.0	8.5	10.8	-2.3
6	6.3	3.5	2.8	10.6	10.8	-0.2
7	7.8	6.2	1.6	11.1	14.6	-3.5
8	6.5	4.3	2.2	12.2	13.0	-0.8
9	6.7	4.8	1.9	10.4	11.9	-1.5
10	6.9	5.1	1.8	12.0	12.1	-0.1
11	4.0	4.8	-0.8	6.9	8.6	-1.7
12	3.8	3.3	0.5	8.9	8.3	0.6
13	6.0	5.1	0.9	10.3	11.3	-1.0
14	4.1	3.9	0.2	6.8	7.9	-1.1
15	3.8	2.5	1.3	7.3	6.3	1.0
16	3.2	2.3	0.9	6.5	6.4	0.1
17	4.4	2.1	2.3	10.1	9.0	1.1
18	2.5	2.3	0.2	9.1	10.7	-1.6
19	5.6	4.9	0.7	10.3	12.2	-1.9
20	3.5	3.0	1.5	8.2	9.4	-0.8
21	3.0	1.9	1.1	4.7	4.3	0.4
22	4.0	3.4	0.6	9.4	12.0	-2.6
23	7.7	5.3	2.4	12.6	13.2	-0.6
24	6.1	4.6	1.5	11.2	14.2	-3.0

* The mean of triplicate estimations of 24 normal subjects by both methods are recorded here.

stopped the reaction and formed the stable final color. This same procedure was followed using the same urine containing a fixed amount of added IAA. The results are illustrated in Figure 24. It is apparent that the xanthydrol reaction in urine extracts continued beyond the 5 minute time interval. When the curve for urine (CURVE B) is subtracted from the curve for urine + IAA (CURVE A), Curve C, representing the added IAA, is obtained. Curve C shows that the reaction of xanthydrol with IAA is complete within 5 minutes; the time interval chosen by Weissbach and associates (1). This agreed with previous curves run using pure solutions of IAA. The colorimetric method yielded the same results on urine hydrolyzed for total IAA.

Upon studying the proposed fluorimetric reaction in the same way, reaction time curves shown in Figure 25 were obtained. Here it is clearly shown that no continued reaction occurred as observed for the xanthydrol reaction.

Further evidence for the lack of specificity of the colorimetric procedure was gained from treatment of the urine samples on ion exchange columns. Previous work with columns has shown that Dowex-1 in the chloride form would absorb IAA from solution but could not again be removed. It was surmized that if a column could be "overloaded" with respect to the non-specific xanthydrol positive urinary constituents, but not so overloaded that IAA would appear in the effluent, a positive indication of non-specificity of the colorimetric method would result.

It was found that if a column 15 mm. long by 8 mm. in

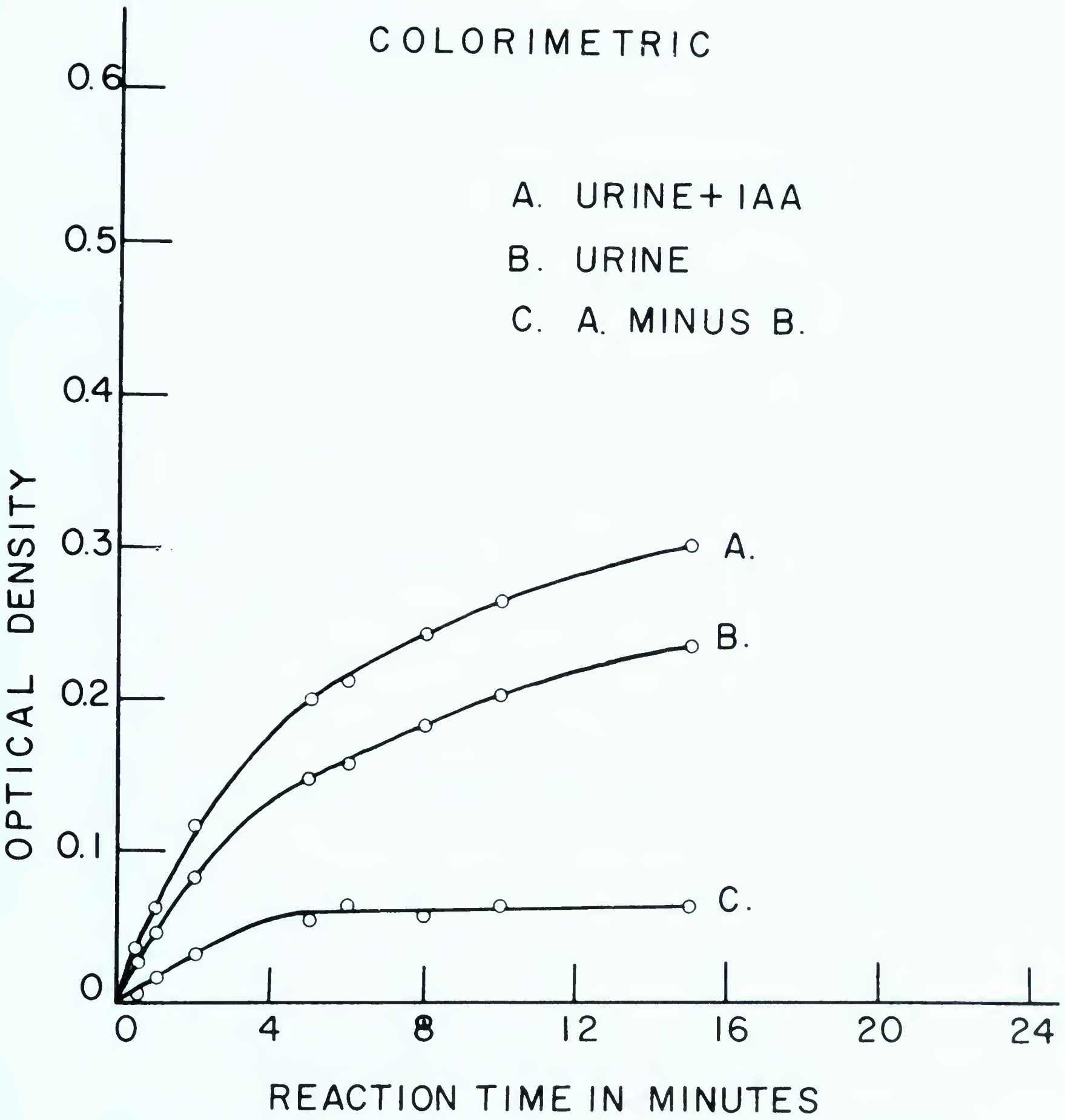


Fig. 24. Xanthydrol reaction time curves for the colorimetric technique of Weissbach, et al.(1).

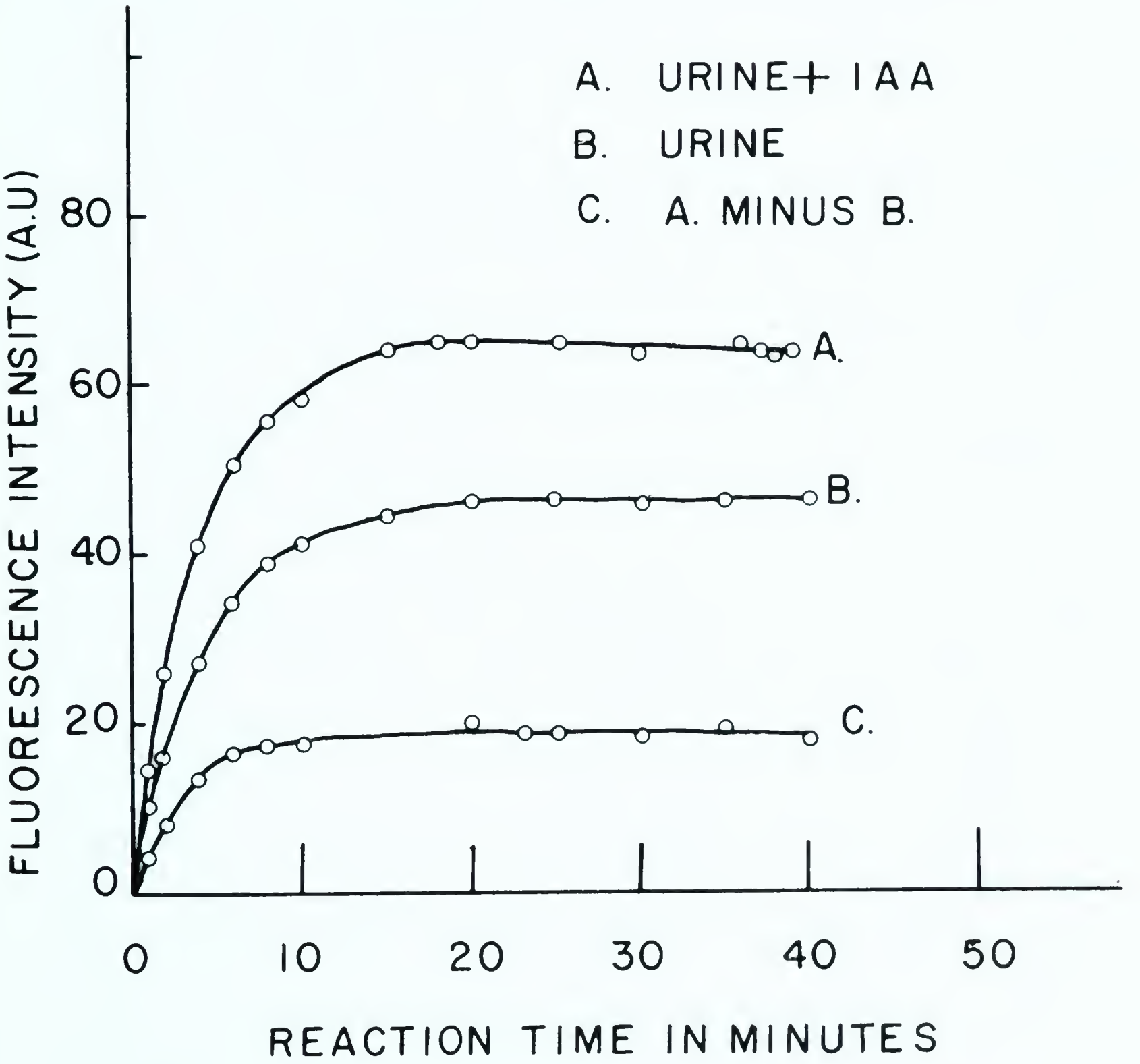


Fig. 25. Reaction time curves for the present fluorimetric technique.

diameter was used, all IAA present in 12 ml. of urine could be retained on the column but did not retain all of the non-specific xanthydrol reacting substances present. Values obtained for several urine samples ranged from approximately 0.25 to 1.3 μg./ml. by the colorimetric method and zero for the fluorimetric technique.

Referring to the xanthydrol reaction of Dickman and Crockett (29), which was modified by Weissbach, et al. (1), it was found that this was reported as a non-specific reaction. Xanthydrol reacted with tryptophan, serotonin, IAA, indolebutyric acid and several other indolic and non-indolic compounds. In order to determine if any interfering substance actually appeared in the final phosphate buffer extract in the colorimetric procedure, a two-dimensional chromatogram was run for indole positive substances. Due to the probably low sensitivity of Weissbach's, et al. (1) colorimetric reagent for staining chromatograms, it was felt that if a post-tryptophan load urine were used, it would increase the excretion of indolic compounds to detectable concentrations. When this procedure was followed with two post-tryptophan load urine samples, only two xanthydrol spots were detectable. One heavy spot represented IAA and the other was identified as indolelactic acid. Indolelactic acid was extractable in the colorimetric procedure as mentioned before. An indolelactic acid standard run through the colorimetric procedure gave a positive xanthydrol reaction.

When the colorimetric and fluorimetric procedures were compared using the two post-tryptophan load urine samples,

the following results were obtained:

Colorimetric:	Sample A.	Free IAA = 172 μg./ml.
		Total IAA= 204 μg./ml.
	Sample B.	Free IAA = 44.8 μg./ml.
		Total IAA= 43.2 μg./ml.
Fluorimetric:	Sample A.	Free IAA = 143 μg./ml.
		Total IAA= 169 μg./ml.
	Sample B.	Free IAA = 32.6 μg./ml.
		Total IAA= 36.2 μg./ml.

Thus the colorimetric method yielded readings approximately 20 to 40% greater than the fluorimetric method. A portion of this difference is probably due to indolelactic acid as suggested above.

It will be noted that in these two samples the total IAA by the fluorimetric method is less than that of the colorimetric method by a substantial margin. In Table X it was shown that the total by the fluorimetric method was significantly greater. This seeming discrepancy is explained by the fact that in post-tryptophan load urine, the IAA is present largely in the free form, as can be seen in the relatively small differences between the free and total values shown above. With low concentrations of conjugated IAA, probably very small amounts of IAA were destroyed in estimating the total by acid hydrolysis in the colorimetric method. The specificity of the fluorimetric method would thus become more apparent and reflect the observed lower values for total IAA estimations.

4. IAA VALUES OBTAINED FOR SEVERAL CONDITIONS OF MALABSORPTION

It is generally accepted that all patients with malabsorption have elevated fecal fat levels. Associated with this increase of fecal fat is an apparent increase or change in the intestinal flora (3). As IAA can be formed from the action of intestinal bacteria on tryptophan, it is possible that a correlation between fecal fat and urinary IAA levels may exist. If such a correlation could be established, it would be possible, and advantageous, to discard the fecal fat test in favor of determinations of urinary IAA. The fecal fat test requires four days (3 days for the collection of samples plus a day for analytical work), whereas urinary IAA can be estimated in 1-2 hours in urine collected during a 24-hour period. Even if no correlation were found to exist, the measurement of urinary IAA might still be useful in studies of the malabsorption syndrome, either as a measure of abnormal bacterial activity in the intestine due to stasis (11, 12, 30), or to assess the progress of treatment (31).

There are a variety of methods available for the diagnosis of intestinal malabsorption. Many of them are difficult to interpret and some are too complex for routine application. Of 20 tests discussed in a recent review by Hunter and Prevatt (32), only three are recommended. These are the D-xylose absorption test, fecal fat estimation and intestinal biopsy. These three procedures were carried out on the patients reported in this study to provide a basis to assess the value of the measurement of IAA excretion in the diagnosis of malabsorption. Serum carotene levels were also estimated, since

this procedure is still widely used.

The presence of abnormal amounts of fecal fat is thought to be a secondary effect, that is, it is not the cause of malabsorption but is only a means of detecting the disorder (33,34). The efficiency of fat digestion and absorption is reflected in the amount of fecal fat. The xylose test is based upon the fact that following the ingestion of D-xylose, it is absorbed from the gut and about 25% of the administered dose appears in the urine within 5 hours. The test is useful in differentiating between steatorrhea of pancreatic origin and idiopathic steatorrhea.

Recently, Ross and Nugent (35) have clarified the confusing array of terms used to describe cases of malabsorption. The following terms have been used in the literature: sprue, celiac disease (childhood and adult forms), idiopathic steatorrhea, nontropical sprue, tropical sprue, gluten-induced enteropathy, and primary malabsorption. The authors state that the majority of patients so labeled are suffering from gluten-induced enteropathy, a term that more clearly identifies the etiologic nature of this form of malabsorption. The term sprue, it was suggested, should be reserved exclusively for the tropical form of malabsorption, as seen in Hong Kong, Puerto Rico and similar areas and whose dominant feature is folic acid deficiency.

Using the fluorimetric technique, total IAA excretion was measured in patients available who exhibited an increased excretion of fat in the stool. Results are given in Table XI.

Based on the case record diagnosis, they included the following: 4 cases of non-tropical sprue, 2 cases of regional ileitis, 1 case of mild steatorrhea following gastrectomy and 1 case undiagnosed. Each IAA value is a mean of triplicate determinations. The data included results of other tests run concurrently, i.e., fecal fat, xylose tolerance, and serum carotene.

It is apparent that there is no correlation between fecal fat content and total IAA excretion. Nor is there any correlation with the xylose tolerance test and serum carotene levels. Normal ranges for each test are:

Total IAA	=	1.5 to 18.4 mg./24 hrs.
Xylose tolerance	=	more than 3 g. excreted in 5 hrs. after a 25 g. oral dose.
Serum carotene	=	more than 50 μg./100 ml.
Fecal fat excretion	=	not more than 5 g./day on 100 g. fat intake.

The first 4 cases reflect this lack of correlation and are typical of others not reported here. The next 2 cases show high fat, high IAA excretion, and 2 cases with high fat, normal IAA excretion. It was noticed from the case histories that most patients with malabsorption had and were receiving a battery of drugs including antibiotics, monoamine oxidase inhibitors and steroids. Possibly the formation and excretion of IAA was affected by some of these. The drugs included tetracycline, Parstellin*, Prednisone**, and Salazopyrine***. One case (J.M.)

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* A monoamine oxidase inhibitor.
** A steroid derivative.
*** A "sulfa" derivative.

TABLE XI

A comparison of total IAA values with several other tests in the malabsorption syndrome

Subjects and diagnosis	Date	Tests and results			
		IAA (mg./24 hrs.)	fat in feces (g./24 hrs.)	xylose tolerance (g./5 hrs.)	serum carotene (µg./100 ml.)
J.L. (Non-tropical sprue)	12.4.63	7.9	20.1	-	14
	14.4.63	.....	on gluten-free diet	.....	
	20.4.63	6.0	6.0	1.2	46
F.J. (Non-tropical sprue)	15.11.61	-	29.9	-	20
	28.11.61	26.1	-	-	-
R.Mc. (Mild steatorrhea)	22.3.62	-	13.6	3.1	108
	3.4.62	10.9	-	-	-
M.B. (Undiagnosed)	23.8.63	138	14.8	-	7
	24.8.63	87	-	-	8
	25.8.63	114	-	-	5
J.M. (Non-tropical sprue)	24.4.62	.....	on Parstellin to 26.5.62	.....	
	1.6.62	-	-	0.6	9
	6.6.62	14.5	34.6	-	-

continued ....

TABLE XI Continued

Subjects and diagnosis	Date	Tests and Results			
		IAA (mg./24 hrs.)	fat in feces (g./24 hrs.)	xylose tolerance (g./5 hrs.)	serum carotene (µg./100 ml.)
E.H. (Regional ileitis)	21.2.62	..... on Salozapyrine to 11.3.62			
	1.3.62	45.2	8.7	11.7	64
	9.4.63	-	-	7.2	14
	13.4.63	..... on tetracycline to 20.4.63			
	15.4.63	-	30.7	-	20
	19.4.63	25.2	-	-	-
	26.4.63	..... on Parstellin & Prednisone to 4.5.63			
	4.5.63	..... on tetracycline to 4.6.63			
	6.5.63	-	29.5	-	-
	16.6.63	13.0	-	-	-
	23.6.63	14.0	-	-	-
		..... on Prednisone			
	19.8.63	8.4	38.6	-	-
A.Mc.(Regional ileitis)	17.4.63	-	-	4.6	-
	6.5.63	-	21.8	-	20
	7.5.63	..... on tetracycline			
	15.5.63	15.6	18.3	-	-
	16.5.63	..... off tetracycline			
	18.5.63	21.3	-	-	-
A.P. (Non-tropical sprue)	16.8.62	38.0	34.0	9.4	18
	1.9.62	..... on gluten-free diet			
	3.9.62	12.7	-	-	-
	11.10.62	-	4.3	5.2	13
	5.11.62	-	-	-	68

was found to have been on Parstellin for some time before admission to the hospital. This was a typical case of malabsorption with a high fecal fat and low carotene level and yet the IAA excretion was normal.

One subject (E.H.) was placed on all 4 drugs at different times and the results obtained are detailed in Table XI. On first admission to the hospital in 1962, the patient was on Salazopyrine. He had an abnormal IAA excretion of 45.2 mg./24 hrs., his fecal fat was slightly elevated and his serum carotene and xylose tolerance tests were normal. The patient was not available for other work until re-admission in 1963, when unfortunately, he was placed on tetracycline therapy before an IAA estimation could be performed. Fecal fats had reached the very high level of 30 g./day, but possibly due to the effect of tetracycline, his IAA excretion was only slightly elevated above the normal at 25 mg./day. The patient was then placed on Parstellin and Prednisone therapy for approximately 1 week and then again on tetracycline for 1 month. Following this, his urinary IAA excretion dropped to 13 mg./day. The patient was then placed on Prednisone alone for a period of time and a further drop in IAA excretion was observed. Throughout the fecal fat remained at a high, abnormal level, apparently not improved by drug therapy.

The effect of tetracycline was further investigated in another patient (A.Mc.) who was also on tetracycline therapy before the first IAA estimation could be performed. A normal value of 15.6 mg./24 hrs. was obtained. The fecal fat and serum carotene values were abnormal. On removal of all drugs

for 2 days, IAA excretion increased to 21 mg./day. It is difficult to say whether this increase was significant since some individuals exhibit a considerable variation in a day to day IAA excretion (3). This patient was discharged before further work could be performed.

A high excretion of both IAA and fecal fat is observed in patient (A.P.). Both values dropped to normal when the patient was placed on a gluten-free diet. A correlation between IAA and fecal fat excretion existed in this case.

A patient with a high IAA excretion was selected for further study of the effect of Salazopyrine and Parstellin on IAA excretion. The diagnosis was regional ileitis. Parstellin was given for 5 days, then Salazopyrine for the next several days. Table XII shows that total IAA excretion remained elevated during Parstellin administration, but dropped following Salazopyrine therapy. Again discharge of the patient prevented further study.

Although the administration of drugs to patients suffering from malabsorption was not under our control, it would appear that certain drugs affect IAA formation and excretion. This might explain the disagreement in results between Marko and Gerrard (4), who reported normal values in malabsorption, and Weissbach and associates (1) and Haverback and associates (3), who reported distinctly elevated values. Clinicians generally feel that drug therapy is of aid in malabsorption (31) and it is quite likely that most IAA excretion values in the literature were obtained under these conditions. If IAA excretion is affected by drugs, the usefulness of the test is limited.

TABLE XII

The effect of Parstellin and Salazopyrine on IAA excretion

	Day	Total IAA mg./24 hrs.
Parstellin	1	34.7
	2	52.6
	3	39.0
	4	51.0
	5	70.5
Salazopyrine	6	--
	12	21.0

5. IAA VALUES OBTAINED FOR OTHER DISEASE STATES

a. Phenylketonuria.

Since there is a great interest in the study of indoles in mental disease (5,6), 2 cases of phenylketonuria were therefore included in this study.

When a routine fluorimetric estimation was performed on two samples of urine from phenylketonuria patients, very low readings for the internal standard were obtained. It is known that in the urine of untreated cases of phenylketonuria, high concentrations of phenylalanine and phenylpyruvic acid are found. It was decided, then, to determine whether these substances were responsible for the high quenching effect observed.

A solution of each substance was prepared in de-ionized water at a concentration of 50 mg./100 ml. One half milliliter of each solution was pipetted into separate extraction tubes. To each tube was then added 0.5 ml. of IAA standard (10 µg./ml.) and 1.0 ml. of de-ionized water. A third tube was set up with only the standard present. These were extracted with 10 ml. $CHCl_3$ and the fluorescence intensity determined in the usual manner. The results are shown in Table XIII. Phenylpyruvic acid is clearly the inhibitor.

No difficulty was found in overcoming this excessive quenching because the IAA concentration was found to be high and allowed for considerable dilution of the specimens. The urine samples were diluted 5 and 10 times for the 2 subjects

studied. No changes in instrument sensitivity settings were necessary. This decreased the quenching effects of phenylpyruvic acid to low levels. The values obtained were 56.0 and 26.6 mg. IAA/g. creatinine. Since the normal range was established at 2.9 to 12.0 mg. IAA/g. creatinine, the IAA excretion values for phenylketonuria were greatly elevated.

TABLE XIII

The effect of phenylalanine and phenylpyruvic acid on fluorescence intensity

Sample*	Fluorescence (a.u.)
(1) IAA	41
(2) IAA + phenylalanine	41
(3) IAA + phenylpyruvic acid	12

* See text for details of sample preparation.

b. Muscular dystrophy.

Six patients suffering from various forms of muscular dystrophy were studied. It was felt that since Weissbach, et al. (1) reported elevated values in several forms of muscular disorder, perhaps IAA estimations could have a place in muscular dystrophy studies.

The values obtained for total IAA are given in Table XIV and were expressed both as mg. IAA/24 hrs. and mg. IAA/g. creatinine.

TABLE XIV

Urinary excretion of IAA patients with muscular dystrophy

Subject	Age (Yrs.)	Diagnosis	Creatinine g./24 hrs.	Total IAA	
				mg./ 24 hrs.	mg./g. creatinine
M.S.	41	Normal (carrier)	3.47	25.0*	7.2
G.S.	13	Pseudohyper-trophic	0.64	7.9	12.2
G.P.	5	Pseudohyper-trophic	0.20	5.3	27.2
W.R.	51	Normal (brothers have early myopathy)	1.81	17.7	9.8
N.R.	37	Possible early myopathy	1.46	20.1	13.8
M.C.	46	Myotonia dystrophica	0.46	5.9	12.9

* Abnormal values are underlined.

Results for IAA excretion based upon creatinine excretion may not be valid in patients with muscular dystrophy, as in this disease creatinine excretion is altered due to muscle wasting. Thus creatinine excretion may not be a reliable gauge of urine output in this condition.

V. CONCLUSIONS

1. A rapid, specific procedure for the quantitative estimation of IAA in urine has been developed. The method will measure as little as 20 nanograms and this may prove useful for serum and tissue studies.

2. It has been confirmed that IAA excretion is increased in some cases of malabsorption. This does not always coincide with fecal fat levels and thus this measurement cannot replace fecal fat measurements for assessment of the malabsorption syndrome.

3. It would appear that drugs used in the treatment of malabsorption affect the formation and excretion of indole-3-acetic acid. Lack of attention to the effect of drugs might be the reason for conflicting results in studies of indole excretion in malabsorption.

4. Increased excretion of IAA was observed in two cases of phenylketonuria. The significance of this is unknown, but may be of interest in that alteration in tryptophan metabolism has been reported in some mental disorders.

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